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EDITORIAL

Treatment of peri-anal fistula in Crohn's disease

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Abstract

Anal fistulas are a common manifestation of Crohn's disease (CD). The first manifestation of the disease is often in the peri-anal region, which can occur years before a diagnosis, particularly in CD affecting the colon and rectum. The treatment of peri-anal fistulas is difficult and always multidisciplinary. The European guidelines recommend combined surgical and medical treatment with biologic drugs to achieve best results. Several different surgical techniques are currently employed. However, at the moment, none of these techniques appear superior to the others in terms of healing rate. Surgery is always indicated to treat symptomatic, simple, low intersphincteric fistulas refractory to medical therapy and those causing disabling symptoms. Utmost attention should be paid to correcting the balance between eradication of the fistula and the preservation of fecal continence.

Key words: Fistula; Crohn's disease; Perianal fistula; Surgery; Surgical treatment; Seton; Anal fistula treatment

Core tip: Treatment of anal fistulas in Crohn's disease is a challenging clinical problem. Although several studies have been published, a consensus on treatment strategy has not yet been achieved. Clinical experience suggests that treatment should be determined according to the type and clinical behavior of the fistula. Asymptomatic fistulas should not be treated, while symptomatic ones could benefit from combined medical and surgical treatment. Surgery can vary from simple drainage and setonage, to more complex and sophisticated procedures. The overall aim of the surgical procedures is fistula healing without compromising fecal continence.

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INTRODUCTION

Crohn's disease (CD) causes inflammation of the lining of the digestive tract, which can lead to severe complications. Inflammation caused by CD can involve different areas of the digestive tract, including the perianal region and the colon (in about 30% of patients). The incidence of perianal fistulas in CD patients ranges from 11 to 38%, and the frequency has been shown to increase with disease duration, being more frequent in patients with colorectal involvement. The etiology of perianal

fistulas in CD remains unclear, but it is often indicative of a more aggressive disease[1-5]. The external openings of the sinus tracts are mostly localized in the perianal region, but can also be detected in the buttock, groin, vulva or scrotum. CD fistulas should always be regarded as complex, even though a simple and single sinus tract is the only finding in many instances. There are several classifications of fistula-in-ano according to current European guidelines[1], although the Parks' classification[6] is currently used. Four groups of fistulae are recognized, according to their relation with the sphincter muscle: (1) extrasphincteric, extending from an internal opening in the bowel proximal to the anus; (2) intersphincteric, traveling along the intersphincteric plane to the perianal skin; (3) transsphincteric, encompassing a portion of the internal and external sphincter; and (4) suprasphincteric, encompassing the entire sphincter apparatus.

CLINICAL MANIFESTATION

Symptoms can vary, including anal pain, purulent discharge and incontinence, and can be associated with high morbidity and an impaired quality of life. This issue assumes particular relevance in younger patients[1]. Perianal disease may develop before the appearance of gastrointestinal symptoms, even several years before the diagnosis of CD[1,3]. The natural history is characterized by a chronic relapsing course, and it may be complicated with perianal sepsis. The recent use of biologic treatments, coupled with a surgical approach, has significantly changed the natural history of perianal disease.

DIAGNOSIS AND ASSESSMENT

A rectosigmoidoscopy is always mandatory when assessing perianal disease in patients with CD, as the presence of rectosigmoid involvement of the lesions is predictive of a more aggressive course[1]. The gold standard for assessing a fistula-in-ano is the exploration of the anal canal and distal rectum under anesthesia (EUA)[1,3,7]. The aim of this procedure is to find the internal orifice and to classify the fistula according to its relationship with the sphincteric apparatus. EUA can be also accompanied by drainage of abscesses and fistula treatment, thus allowing the use of immunomodulators. As also stated by the current European guidelines[1], EUA is considered the gold standard only in the hands of an experienced surgeon.

Additional diagnostic modalities useful for assessing perianal disease in CD include magnetic resonance imaging (MRI) or transanal endoscopic ultrasound (EUS). The sensitivity and specificity of both techniques are lower than EUA and an experienced radiologist or gastroenterologist, respectively, are required in order to achieve a reliable assessment of the perianal lesions[1,8,9]. Pelvic MRI is accurate and non-invasive, although patients' compliance or obesity may not allow its use in a subgroup of patients[10-12]. Moreover, in contrast to transanal EUS, pelvic MRI does not allow the visualization of the rectal mucosa. This represents a major limitation in the assessment of perianal disease in CD, as rectal involvement is associated with a worse outcome of perianal disease. Nevertheless, MRI-based scores can be derived to assess the activity of CD, based on the extent and severity of intestinal inflammation, post-operative recurrence and perianal disease[13]. The accuracy of EUS is comparable to pelvic MRI for assessing perianal CD, and additionally also allows the assessment and histological examination of the rectal mucosa[8-10,12]. However, transanal EUS cannot be performed in the presence of rectal stenosis.

The few studies comparing the sensitivity, specificity, and negative and positive predictive values of these three diagnostic modalities support EUA performed by an experienced dedicated surgeon as the gold standard, which allows not only a proper assessment, but also a local treatment of the disease. The combined use of the three procedures is thought to represent the best modality to assess perianal disease in CD[1,10,12]. Nevertheless, the high cost of this approach limits its use in clinical practice. Therefore, even in referral centers, EUA by an experienced surgeon is often the chosen method, particularly in symptomatic patients requiring surgical drainage or treatment. For asymptomatic patients, local availability of either pelvic MRI or EUS should determine the choice for assessing perianal disease in CD. MRI and EUS can also be used in order to assess the response to biologic therapies, including anti-tumor necrosis factor (TNF) monoclonal antibodies.

The usefulness of computed tomography with intravenous contrast is limited to the visualization of abscesses in the ischiorectal fossa. Additionally, fistulography is not recommended for assessing perianal disease[1].

MANAGEMENT

Management of perianal CD is a relevant clinical issue, as this condition is characterized by a recurrent course and relapses after temporary fistula closure. The aim of treatment is to reduce symptoms, prevent or treat complications, induce fistula closure and improve the quality of life. The recent use of anti-TNFs has been shown to significantly improve the course of perianal disease in CD. Treatment should be determined according to the type and severity of the fistula. However, treatment is related to symptoms, and asymptomatic perianal fistulas should not be treated. Furthermore, for treatment of luminal CD, a multidisciplinary approach including a dedicated gastroenterologist, surgeon and radiologist is advisable. For symptomatic fistulas, antibiotic treatment is recommended before treatment with immunomodulators and/or anti-TNFs[5,14,15].

Although multicenter, randomized placebo-controlled studies including large series of patients are currently lacking, current evidences suggest that the use of metronidazole (750-1500 mg/d), ciprofloxacin (1 g/d) or both, shows efficacy in terms of temporary relief of symptoms and in treating local complications. Antibiotic-therapy has

not been shown to be efficacious in terms of fistula closure[1,5,14,15]. However, a proper evaluation of the optimal treatment strategies has been constrained as trials on the use of metronidazole and ciprofloxacin for perianal CD utilize different study designs, with different antibiotic dosages, combinations and durations of treatment.

Azathioprine (AZA; 2.0-2.5 mg/kg, by mouth) or 6-mercaptopurine (6-MP; 1.0-1.5 mg/kg, by mouth) are immunomodulators indicated for perianal fistulas in CD patients failing treatment with antibiotics with or without previous surgical treatment[1,16,17]. The presence of a perianal abscess should be excluded by using EUA and/or EUS or pelvic MRI and it requires surgical drainage and often the placement of a draining seton[1]. The efficacy of AZA and 6-MP requires at least 3-6 mo of treatment. In patients failing treatment with AZA or 6-MP, biologics therapy using anti-TNFs such as Remicade or adalimumab is recommended, as they have been shown to induce fistula closure in up to 36% of patients at 56 wk[1,18-21]. Remicade (5 mg/kg, iv) at 0, 2, and 6 wk, followed by maintenance treatment every 8 wk, or adalimumab (160, 80, or 40 mg, sc) at 0, 2, and 4 wk, followed by maintenance treatment every 2 wk currently represent the more effective treatments for inducing fistula healing in CD. Interestingly, MRIs show that anti-TNF treatment, though able to induce the healing of the external orifice, may not be sufficient to close the fistulous tract[22]. This observation accounts for the relapse of local discharge and for the development of perianal abscesses along the fistulous tract. Preliminary observations suggest that local injection of Remicade in the fistulous tract may be useful in inducing fistula healing[23,24]. However, these are preliminary observations that need to be confirmed by randomized controlled trials.

Fistulous tracts have been treated by injection with biologic glues. Injection of a novel cyanoacrylic glue using image-guided percutaneous techniques has been used to treat post-surgical fistulas, but results need to be confirmed[25]. In a randomized controlled trial, injection of the fistulous tract with fibrin glue demonstrated a 38% success rate[26]. The failure rate associated with fibrin glue injection was attributed to the difficulty in ensuring that the glue remained in the fistula tract[27]. Discrepant findings concerning biologic glues have been reported in a meta-analysis[28]. As for local injections, these observations need to be confirmed by multicenter studies including a larger number of patients. Additional preliminary observations in a preclinical study suggest that autologous fibroblasts added to the collagen glue may improve the outcome of perianal disease when compared with patients treated with the glue only[27]. The addition of autologous fibroblasts to the collagen glue has been suggested to reduce the slippage of the glue from the fistulous tract[28].

SURGICAL TREATMENT

Surgical treatment of fistulas can vary from simple drainage to more complex and sophisticated procedures. The surgical approach depends upon the type of fistula and its anatomical extent. It is important to remember that in CD, only symptomatic perianal fistulas need surgery. Some fistulas can be surgically excised and a cure achieved, whereas other patients will benefit from symptom palliations. Palliation usually comes in the form of drainage and thereafter, a long-term, comfortable, loose, seton[29].

Surgery should be considered in patients who have simple, low, intersphincteric fistulas or fistulas refractory to medical therapy and those who have severe or disabling symptoms. However, surgery should not be performed in patients with active proctitis. The goals of surgery are to eradicate the fistula while preserving fecal continence, or to reduce symptoms by making management easier for the patient, such as by transforming a complex fistula into one closer to the anus. Surgical options include long-term setons, cutting setons, fibrin glue, fistula plugs, fistulotomy, fistulectomy, advancement flaps, and proctectomy.

For patients with intersphincteric or low transsphincteric fistulas, fistulotomy is advised and may lead to healing in a significant number of patients. It is not a feasible option when the fistula incorporates a significant amount of the internal and external anal sphincter, as occurs with high transsphincteric fistulas. To avoid poor healing and higher recurrence risk, good patient selection is mandatory and surgery should be delayed whilst optimizing the treatment of active proctitis[30].

Advancement flaps can be used as a sphincter-preserving technique for some higher fistulas in CD. The transanal mucosal advancement flap involves creating a flap of mucosa and a portion of the muscular wall of the rectum from around the internal opening of the fistula and into the lower rectum. The internal opening of the fistula is excised from the distal flap, and the flap is sutured to the distal dissection plane to cover the area of the formal internal opening and to create a neo-dentate line. The success rate of advancement flaps, based on a systematic review of more than 2000 procedures (a small subset having CD) is 64%, with incontinence rates of 9.4%[31].

It is our belief and experience that in complex fistulas, the first line of treatment is often a loose non-cutting seton. This option is a safe one; it helps the drainage of the sinus, prevents development of a more complex scenario, and it is mostly well-tolerated. A loose seton may be passed and subsequently, associated treatment with anti-TNF therapy is offered; in this case the healing rate is 47%-79%[30]. A loose seton can be converted, in select cases, in a cutting seton. Cutting setons result in low recurrence rates, but can cause incontinence in up to two-thirds of patients[32].

Apart from the original technique of fistulectomy and fistulotomy, several different approaches have been described to treat perianal fistulas. However, it is important to remember that all these techniques have been designed to treat complex fistulas in otherwise healthy individuals.

Moreover, most of the reported results are from series of fistulas not associated with CD or mixed-case series.

The ligation of the intersphincteric fistula tract (LIFT) procedure is a modern technique based on secure closure of the internal opening and removal of infected tissue through the intersphincteric approach. Essential steps of the procedure include incision at the intersphincteric groove, identification and ligation of the intersphincteric tract close to the internal opening, and removal of the intersphincteric tract. LIFT has been associated with fistula closure rates between 57% and 94%, but higher quality of evidence with longer follow-up is still needed[33,34]. In one study involving four CD patients, the LIFT procedure was successfully combined with the placement of a biosynthetic graft[35]. However, further studies are necessary before the LIFT procedure in any form can be recommended in the treatment of CD perianal fistulas.

The Surgisis® anal fistula plug (Cook Surgical, Bloomington, IN, USA) is a bioabsorbable xenograft made of lyophilized porcine intestinal submucosa. The material is resistant to infection, provides no foreign body or giant cell reactions, and becomes repopulated with patient's cells and tissues over a period of three months. The success rates reported for this plug are variable and range from 13% to 86%[36]. A theoretical advantage to using this plug is that the operative technique involves suturing the plug to the internal anal sphincter at the site of the internal opening, which keeps the material in place and allows time for ingrowth.

A promising technique involves the use of stem cells to stimulate fistula closure. Garcia-Olmo *et al*[37] performed a randomized clinical trial comparing adipose-derived stem cells injected into the rectal mucosa and fibrin glue. The fistula closed in 71% of patients in the stem cell group compared with 16% in the fibrin glue group.

The continued search for innovative techniques, have led to the use of video-assisted anal fistula treatment (VAAFT). An initial report by Meinero and Mori showed promising results with the VAAFT technique in patients with complex anal fistulas[38]. This technique allows a direct visualization of all the sinus tracts with the aim to reduce recurrence. Schwandner combined VAAFT with advancement flaps and showed a high identification rate of occult side tracts with encouraging short-term healing rates[39]. However, only a small percentage of the VAAFT series included CD patients, thus the technique needs further evaluations with increased sample sizes and long-term follow-ups.

In our unit, we are currently assessing the feasibility of outpatient exploration of the anal canal and distal rectum. Preliminary findings suggest that outpatient explorations is feasible in the vast majority of patients and that it is possible to perform a complete treatment in over 80% of patients[40]. We strongly believe that the key to success is a multidisciplinary approach; the patients were well-known to all the members of the team, and were in regular follow-ups at our Inflammatory Bowel Disease Referral Center. Patients with perianal CD are all seen in a joint clinic by the referring gastroenterologist and surgeon, and a specialist nurse trained as a theater nurse is also always present. Finally, a diverting stoma may become necessary in cases of recurrent fistulas refractory to medical treatment or in cases with severe urogenital complications, fecal incontinence or severe proctitis. It is our experience, however, that a stoma will very rarely be reversed and in many instances, a proctectomy will follow.

CONCLUSION

Perianal disease is a troublesome condition for both patient and surgeon. Management of this condition involves a delicate balance between the eradication of the fistula and preservation of fecal continence. It requires a multidisciplinary collaboration between a dedicated gastroenterologist, surgeon and radiologist. Surgery should be conservative and patient collaboration and understanding of the scope of surgery is crucial for optimal management.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Surgical treatment of ulcerative colitis: Ileorectal *vs* ileal pouch-anal anastomosis

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Core tip: Ileal pouch-anal anastomosis (IPAA) is the most commonly performed procedure for treatment of UC patients refractory to medical therapy. However, IPAA carries on its own risks. Recently, some authors have proposed ileorectal anastomosis (IRA) as a valid surgical alternative to IPAA. IRA is an easier operation than IPAA associated with low complication rates and comparable long-term functional results. This manuscript reviews the pros and cons of both procedures and compares results.

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Abstract

Total proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the current gold standard in the surgical treatment of ulcerative colitis (UC) refractory to medical management. A procedure of significant magnitude carries its own risks including anastomotic failure, pelvic sepsis and a low rate of neoplastic degeneration overtime. Recent studies have shown that total colectomy with ileorectal anastomosis (IRA) has been associated with good long-term functional results in a selected group of UC patients amenable to undergo a strict surveillance for the relatively high risk of cancer in the rectum. This manuscript will review and compare the most recent literature on IRA and IPAA as it pertains to postoperative morbidity and mortality, failure rates, functional outcomes and cancer risk.

INTRODUCTION

The main goals of surgical treatment for ulcerative colitis (UC) are not only to alleviate symptoms and minimize cancer risk but also to obtain good functional outcomes and improve quality of life. Until the 1950s total proctocolectomy with end-ileostomy (TPC) was the only available procedure for UC patients failing medical management.

In the 1940s reports of subtotal colectomy with ileorectal anastomosis (IRA) as an alternative to TPC in selected patients were first published[1]. During the 1950s and 1960s, Aylett[2] became the leading proponent of this procedure describing it as a way to avoid a permanent stoma. At that time IRA represented a valid alternative to TPC in high selected patients with minimal rectal inflammation. It was a less invasive operation, performed in one

Table 1 Morbidity and mortality after ileorectal anastomosis

Series	Period	*n*	Anastomotic leak (%)	Proctitis (%)	Need for proctectomy (%)	Overall morbidity (%)	Mortality (%)
da Luz Moreira *et al*[11]	1971-2006	86	2.3	28.0	53	8	0
Leijonmarck *et al*[13]	1955-1984	51	3.9	45.1	57	16	4
Pastore *et al*[15]	1974-1990	48	4.4	10.4	17	22.9	0
Börjesson *et al*[16]	1997-2003	32	3.1	9.3	12	28	0
Grundfest *et al*[17]	1957-1977	89	9.1	11.2	21	16.1	0
Elton *et al*[18]	1990-1999	18	5.6	11.0	17	22.2	0
Andersson *et al*[19]	1992-2006	105	2.8	8.6	13.3	12.4	0
Lepistö *et al*[22]	1978-2000	20	-	45.0	35	-	0
Oakley *et al*[23]	1960-1982	288	-	41.0	55.2	-	4.2

stage and not requiring pelvic dissection with the associated risk of sexual dysfunction[3-5].

In 1978, Parks *et al*[6] described an ileal pouch-anal anastomosis (IPAA). Since then, IPAA has become the procedure of choice for patients affected by UC with excellent long-term functional results, low risk of persistent cuff inflammation or neoplastic degeneration in the retained rectum[7,8]. Consequently, many surgeons have abandoned IRA in favor of IPAA and TPC has remained an option for patients not candidates for IPAA. The counterargument is that IPAA, as a major procedure, carries its own risks including anastomotic failure and pelvic sepsis that could result in poor pouch function, pouchitis and infertility in young women, as well as pelvic nerve damage and portal vein thrombosis[3-5,9,10]. In addition few cases of cancer have been reported arising not only in the anal transitional zone but also in the pouch itself[7,8].

Interestingly, recent series[11-14] of selected UC patients undergoing IRA showed long-term functional results similar to IPAA.

The aim of the current study was to review and compare the most recent literature on IRA and IPAA as it pertains to postoperative morbidity and mortality, failure rates, functional outcomes and cancer risk. It will help surgeons to provide a tailored treatment for UC patients.

ILEORECTAL ANASTOMOSIS

Chronic UC begins in the rectum and extends proximally in a continuous fashion. The severity of the disease also seems to be higher distally with the exception of fulminant pancolitis presentation. However, distal disease is sometimes alleviated by topical treatment and patients with minimal rectal involvement and no dysplastic changes in the rectum could be considered for IRA. Furthermore, an adequate rectal compliance and a normal anal sphincters function are critical for good long-term results. These functions can easily be assessed by digital rectal examination but more accurately by rigid/flexible proctoscopy and manometry. Patients with poor sphincter function, severe rectal disease, and non-distensible rectum should not be offered an IRA. On the contrary, patients with colitis associated colorectal cancer and advanced metastatic disease may benefit from an IRA because of their short life expectancy and the palliative nature of their treatment.

Several studies[11,13,15-17] have shown IRA for UC to be safe, with low postoperative morbidity and mortality. During the years, overall morbidity has been reported between 8% and 28% and mortality between 0% and 4% (Table 1). These studies including the work of Elton *et al*[18] and Andersson *et al*[19], focused their attention on postoperative complications including small bowel obstruction, anastomotic leak and abdominal abscess. The fatal events were due to anastomotic leak and subsequent sepsis and to a pulmonary embolism.

The majority of published data has included mainly primary anastomosis with leak rates ranging from 2% to 9%[11,13,15-20]. Diverting ileostomies have been utilized in selective cases at the surgeon discretion. Turnbull[21] suggested that preservation of grossly involved rectosigmoid colon was the main cause of IRA failure. In his opinion an anastomosis at 6 cm or less above peritoneal reflexion improved rectal inflammation during the first months and reduced the likelihood of IRA failure.

IRA does not involve extensive pelvic dissection, unlike IPAA or TPC, minimizing the risk of sexual and urinary dysfunction. Hence, higher fertility rates may be expected in IRA patients compared to IPAA although definitive studies providing evidence for better fertility rates in UC patients are lacking. Thus, colectomy with IRA could be considered when treating women in their reproductive age[20].

Some authors[2,11,13,15,18,22] have shown acceptable long-term success rate after IRA. Aylett[2] in 1966 reported on a total of 300 cases operated on over a ten-year period with only 7% failure rates. Lepistö *et al*[22] and Pastore *et al*[15] reported a cumulative probability of having a functioning IRA at five years of 84%. Elton *et al*[18] had 88% success in their 18 patients, but the follow-up in that study was shorter. Ten year cumulative success (69%) in Lepistö's series[22] was higher than reported by Leijonmarck *et al*[13] (51%) in 1990. At 20-years the current probability of having a functioning IRA has ranged between 46% and 69 %[20]. One recent study proposed by da Luz Moreira *et al*[11], from Cleveland Clinic, compared 22 IRA with 66 IPAA patients matched for age, gender, and follow-up time, including IRAs performed in the past 25 years showed a cumulative probability of having a functioning IRA at 5, 10, 15 and 20 years of 81, 74, 56

and 46 percent respectively in accordance with previously published work.

In terms of functional results, the da Luz Moreira's series[11] reported six bowel movements per day (range 2-11), 1/22 (5%) night-time seepage and 15/22 (68%) reporting frequent urgency. Leijonmarck *et al*[13] showed four bowel movements per day and none during the night, with 100% of continence (25% of patients are on antidiarrheal medication), after a mean follow-up of 13 years. Elton *et al*[18] had no significant difference between preoperative and 1-year postoperative stool frequency, 11/12 patients had no problems with continence, and three were using antidiarrheal medication. Pastore *et al*[15] described a median number of six bowel movements per day (range 2-20). The median number of nocturnal bowel movements was one (range 0-10) and three patients had more than eight daily stools with frequent soiling and urgency. At the time of follow-up, antidiarrheal medications were taken by 53.3% of patients, whereas 31.3% required low doses of systemic or topical steroids. More than 90% of patients considered that their health status had improved after the operation. Quality of life was improved in 84%.

All the studies above showed that IRA is a safe procedure with an acceptable function and quality of life but unfortunately it is not necessarily a definitive operation, especially for young patients. Specifically, Andersson and colleagues[19] reported an estimated cumulative failure rate of 10.1% and 24.1% respectively at 5 and 10 years. In his series Leijonmarck *et al*[13] had 57% of failure after 13-year follow-up. Pastore *et al*[15] suggested that time between IRA and additional surgery in his series was 3.9 ± 4.7 years. In da Luz Moreira's series[11], 38 patients (44%) continued to have a functioned IRA after a median follow-up of 11 years (range 1-30 years). The rectum was resected in 46 (53%) of 86 patients and the median follow-up between IRA and completion proctectomy was 10 years (range 1-33 years).

The main indication for proctectomy is recurrent proctitis refractory to medical management[11,13,15-19,22,23], followed by dysplasia or cancer, and the development of Crohn's disease. The options for these patients include IPAA, Brooke ileostomy, or a continent ileostomy (Kock pouch). Very Often IPAA can be safely performed in the majority of these patients thus preserving bowel continuity and avoiding permanent fecal diversion[11].

Cancer risk

Mucosal dysplasia is a premalignant pathological state associated with long standing UC[24]. Dysplasia in general is considered an indication for surgery in UC, even though this paradigm is rapidly changing. Epithelial dysplasia of the colon and rectum was graded as mild, moderate, or severe depending on whether the upper one-third, upper two-thirds, or entire glands displayed nuclear anisocytosis and hyperchromatism, as well as loss of nuclear polarity and the normal goblet cell configuration of colonic mucosa. Since dysplastic changes were often patchy, only the highest degree of dysplasia was considered. Johnson *et al*[25] had shown in 1983 that the probability of developing rectal adenocarcinoma after a diagnosis of mild or severe dysplasia in IRA patients reached 42% at nine years from diagnosis. The rate of dysplasia and cancer, in patients with UC, increases with time and leaving the rectum in place contributes to the increased risk. The overall cumulative probability of rectal dysplasia in the retained rectum increases from 9% at 10 years to 25% at 20 years[11]. The overall incidence of rectal cancer after an IRA varies in the literature based on length of follow-up, ranging from 0% to 18%. Grundfest *et al*[17] reported on four patients who developed carcinoma of the rectum during their study period (4.8% at 8-year follow-up), although he estimated the risk of rectal cancer to be 13% at more than 25 years of follow-up. Oakley *et al*[23] found nine patients with rectal cancer in the stump (3.1%) at an 8-year follow-up while Andersson *et al*[19] showed an overall risk of cancer of 1.9% at a 5.4-year follow-up. However, some series reported higher rates of degeneration as Baker *et al*[26] who described, in 1978, a cumulative cancer risk of 6% after 20 years rising to 18% after 35 years in a series of 374 unselected patients. In da Luz Moreira's series[11], the cumulative probability of developing dysplasia and cancer was 7%, 9%, 20% and 25% and 0%, 2%, 5% and 14% at 5, 10, 15 and 20 years respectively. On the other hand, Leijonmarck *et al*[13] and Lepistö *et al*[22] had reported no case of cancer in more recent series at 13 and 18-year follow-up, respectively. Pastore *et al*[15] showed a cumulative probability of remaining free of cancer around 85.5% at 12 years (95%CI: 57.7%-100%).

Most patients who develop rectal cancer in the retained rectum presented at an advanced stage (stage III-IV) suggesting the possibility of a more aggressive biology and making close surveillance imperative[11,27]. For instance, in Baker's study 62% of patients who had developed rectal cancer died within three years of diagnosis. Johnson *et al*[27] reported a total of 10 rectal cancers, 8 of which had either nodal or distant metastases. The patients in the series reported by Oakley *et al*[23] fared better, with just 2 of 9 patients with rectal cancer dying over a 22-year time period. Rectal biopsies taken from multiple sites every 6 to 12 mo are advised following IRA in UC patients. If dysplasia is found, completion proctectomy is indicated. Patients with long standing UC who are not able or willing to undergo surveillance should not be offered an IRA. It is also important to emphasize that colectomy with IRA should not be offered to patients with preexisting dysplasia or cancer due to the increased risk of further neoplastic degeneration[28]. In addiction, the presence of dysplasia or cancer in the resected colon should cause particular concern about the fate of the remaining rectum suggesting that a completion proctectomy would be indicated in these cases. In fact, Oakley *et al*[12] reported on five surviving patients who had cancer in their colonic specimens; three of the five were found on follow-up to have cancer or severe dysplasia in the rectal remnant. Grundfest *et al*[17] described nine patients

Table 2 Morbidity and mortality after ileal pouch-anal anastomosis

Period	No. of studies	No. of patients	Pelvic sepsis (%, 95%CI)	Pouch failure (%, 95%CI)	Pouchitis (%, 95%CI)	Mortality (%, range)
Meta-analysis studies < 2000[32]	43	9317	9.5 (8.2-10.9)	6.8 (5.8-8.4)	18.8 (15.7-22.4)	-
Meta-analysis studies ⩾ 2000[33]	53	14966	7.5 (6.1-9.1)	4.3 (3.5-5.3)	26.8 (21.0-33.5)	0 (0-2.9)

with a colitis-associated colon cancer or severe dysplasia who underwent subtotal colectomy, eight of whom survived; of the eight, five developed severe dysplasia or cancer in the retained rectum.

ILEAL POUCH-ANAL ANASTOMOSIS

Restorative proctocolectomy with IPAA is currently the procedure of choice for the surgical treatment of UC. The main reason for its popularity is its avoidance of a permanent stoma with stable functional results and good quality of life. In over 30 years of its existence, the IPAA has undergone several refinements in the quest of achieving optimal results. Examples include different shapes of the pouch, different anastomotic techniques, use of defunctioning ileostomy and various dissection methods[29-31]. Surgeons have also obtained greater experience and familiarity with the technique, which has also benefited outcomes.

A large body of literature exists on the outcomes of IPAA. Most studies, however, are retrospective cohorts reporting outcomes from a single institution. Due to large variations between studies, an overview is needed for reliably assessment of the IPAA outcomes. A meta-analysis of 43 observational studies, all published before 2000, has provided pooled estimates of complications and functional outcomes after IPAA[32]. This meta-analysis showed a pouch failure risk of 6.8% (95%CI: 5.4%-8.4%), increasing to 8.5% (95%CI: 5.4%-13.2%) when only patients with a minimal follow-up of 5 years were considered[32]. Other pouch related complications were also studied. Pelvic sepsis and pouch fistulas, both major postoperative complications, were observed in 9.5% (95%CI: 8.2%-10.9%) and 5.5% (95%CI: 4.3%-7.0%), respectively. Sexual dysfunction was present in 3.4% (95%CI: 2.7%-4.7%), while pouchitis was reported in 18.8% (95%CI: 15.7%-22.4%).

A recent meta-analysis, including 53 studies published after 2000, showed significant improvements in these results[33]. The overall rate of pouch failure was significantly reduced to 4.3% (95%CI: 3.5%-5.3%), and pouch failure after at least 5 years of follow-up was 4.7% (95%CI: 3.4%-6.4%). An improvement was also seen in most other complications. Pelvic sepsis, pouch fistula and sexual dysfunction were reported in 7.5% (95%CI: 6.1%-9.1%), 4.5% (95%CI: 3.5%-5.7%) and 3.0% (95%CI: 1.7%-5.2%) of patients. The only complication showing a substantial increase was pouchitis, with a rate of 26.8% (95%CI: 21.0%-33.5%).

Thus it seems that the rate of complications after IPAA has declined over time (Table 2). The authors of the meta-analyses have noticed that the decline was largest in the earlier period of the IPAA, but seems to have continued over time. Nonetheless, IPAA remains a complex surgery with substantial risk of morbidity. The high rate of pouchitis is also worrisome, since this complication can affect functional outcomes, quality of life and might also increase risk of dysplasia in the pouch. It should be noted that the meta-analyses discussed above did not distinguish between acute and chronic pouchitis, which is an important distinction in terms of course and health implications[34-36].

Functional outcomes after IPAA were similar in studies published before and after 2000[33]. Average frequency of bowel movements per 24 h was 5.9 (95%CI: 5.0-6.9), of which 1.5 (95%CI: 1.0-2.1) overnight. Mild and severe faecal incontinence were reported in 14.3% (7.3%-25.9%) and 6.1% (2.9%-12.3%) of patients, respectively. The authors conclude that functional outcomes of IPAA may be determined by an intrinsic limitation of the IPAA procedure, rather than growing expertise or technical refinement. This is in line with other studies showing no improvement in functional outcomes based on technical developments, such as type of anastomosis or laparoscopic approach[36,37]. However, most patients consider the functional outcome after IPAA to be highly satisfactory, with good quality of life and social functionality that are comparable to those in a healthy reference population[38,39]. As expected, achieving these adequate quality of life scores was highly correlated with achieving of good functional outcomes[40].

Cancer risk

The IPAA has as an important advantage the removal of the whole colon and virtually the entire rectum as part of the procedure. This minimizes chances of colon and rectal cancer in this high-risk population. A proctocolectomy should be considered almost mandatory when dysplasia is present. Even when only low-grade dysplasia has been identified by colonoscopy, the risk remains substantial. In such patients, studies show a risk of concomitant cancer or high-grade dysplasia of 15% and a 5-year progression rate of up to 54% if not operated on[28,41].

When a double-stapled approach for IPAA is used, a mucosal remnant at the anal transition zone (ATZ) is left in place. The risk of cancer in this area is a matter of controversy. In three series with long-term follow-up focused on this outcome, dysplasia and cancer in the anal transitional zone after stapled pouch surgery was found to be infrequent[7,42,43]. Dysplasia was observed in 8/178

Table 3 Main advantages and disadvantages of ileorectal anastomosis and ileal pouch-anal anastomosis

	IRA	IPAA
Advantages	Easier operation	Lower risk of cancer
	Lower infertility rate	No need for medical therapy
	Lower risk of urinary and sexual dysfunction	Less urgency
	Fewer bowel movements per day	
	Better continence	
Disadvantages	Need for maintenance therapy	Major operation
	Risk of recurrent/persistent disease	Risk of postoperative complications (pelvic nerves damage, pelvic sepsis, portal vein thrombosis)
	Higher risk of neoplastic degeneration	Pouchitis
	Need for strict surveillance	
	More dietary and work restrictions	

IRA: Ileorectal anastomosis; IPAA: Ileal pouch-anal anastomosis.

(4.4%), 7/210 (3.3%) and 0/135 (0%) after at least 10 years of follow-up. In most of these cases, dysplasia developed in the first 2 to 3 years and often disappeared on repeated biopsies. None of the series found cancer in the ATZ after such prolonged follow-up. These data strongly emphasize the extent to which IPAA minimizes the risk of cancer.

The best evidence regarding the development of dysplasia and adenocarcinoma after IPAA can be obtained from a recent study from the Cleveland Clinic[44], in which 3203 patients undergoing an IPAA from 1984 to 2009 were analyzed. Cumulative incidences for pouch neoplasia at 5, 10, 15, 20, and 25 years were 0.9%, 1.3%, 1.9%, 4.2%, and 5.1%, respectively. Overall, 23 patients (0.72%) developed dysplasia, while 11 (0.36%) developed adenocarcinoma of the pouch and/or the ATZ. Risk factors for pouch neoplasia were also evaluated. Preoperative established cancer [hazard ratios (HR) = 13.43, 95%CI: 3.96-45.53, $P < 0.001$] or dysplasia (HR = 3.62, 95%CI: 1.59-8.23, $P = 0.002$) were the only independent factors associated with increased risk of pouch neoplasia. Mucosectomy did not protect against this risk, and the rate of pouch cancer was actually higher after mucosectomy with a rate of 1.3% (6/451) compared to 0.3% (9/2734) after the double-stapled approach. The authors[44] concluded that the risk for neoplasia in patients with UC and IPAA is small, and that it is mainly determined by the presence of preoperative dysplasia or cancer.

Additionally, in a review of literature, 26 published case reports were identified between 1984 and 2008[45]. Certain observations from this review are noteworthy. First, of the 26 carcinomas, 14 (52%) arose from rectal mucosa or from the anal transition zone, while 6 (23%) were from ileal pouch mucosa. Second, adenocarcinomas developed after mucosectomy in 17 patients, and after a double-stapled approach in 8 patients (1 case not reported). Also worth noting, the indication for the IPAA was due to neoplasia in 19 patients (9 cancers and 10 dysplasia) and non-neoplasia in 6 patients. The median time for development of pouch lesions was the shortest in patients operated on for cancer (median 3 years), compared to a median of 6.5 in the other patients. This review is in line with results from the above mentioned study, and further establishes the following conclusions: (1) the low number of reported cases; (2) cancer can develop both after mucosectomy or double-stapled approach; and (3) the close relationship between surgery for neoplasia and development of cancer. The review was not able to estimate the incidence of cancer after IPAA, since the total number of IPAA cases was not stated in most case reports. Branco *et al*[45] did publish their own case as part of this review, which was the first case they observed in a cohort of 520 patients (0.2%) from 1978 to 2008. This percentage is also in line with the Cleveland study[44].

Despite this seemingly small risk, surveillance of selected patients has been recommended by some authors[46,47]. This approach might especially be important in UC patients with dysplasia or cancer present at time of surgery, or patients with retained rectal mucosa and active inflammation (*i.e.*, cuffitis). Also the presence of chronic pouchitis might be a valid indication for surveillance, since this has been associated with increased risk of low-grade dysplasia (odds ratio 13.48, $P < 0.02$), as well as high-grade dysplasia (3/66 *vs* 0/210, $P = 0.01$)[35].

CONCLUSION

In the current era IPAA is the preferred approach for patients with UC requiring surgical treatment. The removal of all diseased mucosa and the lower risk of cancer after IPAA compared to IRA are the main advantages of this technique (Table 3). Therefore, IPAA should certainly be performed when the rectum is actively involved in the disease or when dysplasia or cancer are present in any part of the colon or rectum. Nonetheless, there is still a role for IRA and TPC for selected patients and for patients not candidates for IPAA.

Total abdominal colectomy with IRA is justified in UC patients with normal anal sphincters tone without severe perineal disease, and spared and distensible rectum with no evidence of dysplasia or cancer at the time of intervention. It can be also proposed to young women as a possible interim procedure based on concerns for infertility after IPAA.

The risk of cancer is of particular concern in the comparison between these two techniques. Current evi-

Table 4 Risk of cancer after ileorectal *vs* ileal pouch-anal anastomosis in ulcerative colitis

	Period	*n*	Follow-up average (yr)	Overall cancer rate (%)	Estimated cumulative risk after 20 years (%)
Ileorectal anastomosis					
da Luz Moreira *et al*[11]	1971-2006	86	9	8	14
Leijonmarck *et al*[13]	1955-1984	51	13	0	-
Pastore *et al*[15]	1974-1990	48	6.3	2	14.3[1]
Börjesson *et al*[16]	1997-2003	32	3.5	0	-
Grundfest *et al*[17]	1957-1977	89	8	4.8	5 ± 3.5
Elton *et al*[18]	1990-1999	18	2.6	-	-
Andersson *et al*[19]	1992-2006	105	5.4	-	2.1
Lepistö *et al*[22]	1978-2000	20	18	0	-
Oakley *et al*[23]	1960-1982	288	8.2	3.1	-
Baker *et al*[26]	1952-1976	374	> 10	5.9	6 ± 2
Ileo-pouch anal anastomosis					
Kariv *et al*[44]	1984-2009	3203	± 12	0.4	4
Branco *et al*[45]	1978-2008	520	± 15	0.2	-

[1]Cumulative risk at 12 years (rather than 20).

dence shows a large variation in the reported rates of cancer after IRA from 0% to 8%. For IPAA, this risk is much smaller, and two large series have shown a rate of cancer of about 0.3%. Few studies have calculated the cumulative risk of cancer as well. Similarly, estimated cumulative risk of cancer after 20 years was higher after IRA (6% to 14%) compared to IPAA (4.2%) (Table 4).

Therefore, every patient undergoing IRA should be informed about the risk of recurrent proctitis and cancer in long standing disease. They have to fully understand the need for meticulous surveillance and agree to comply with at least yearly endoscopy with rectal biopsies. Unless these conditions are met, patients should not be offered an IRA. Also, patients with widely metastatic colorectal cancer may benefit from an IRA as a palliative procedure.

Functional results seem to be better after IRA with lower frequency of bowel movements and less night-time seepage but with more urgency compared to patients with an IPAA. The overall quality of life is similar, although the IRA group has significantly more dietary and work restrictions[11].

Finally, TPC still remains the procedure of choice in patients with impaired anal sphincter function and high-risk of pouch failure.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Crohn's disease and growth deficiency in children and adolescents

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Abstract

Nutritional concerns, linear growth deficiency, and delayed puberty are currently detected in up to 85% of patients with Crohn's disease (CD) diagnosed at childhood. To provide advice on how to assess and manage nutritional concerns in these patients, a Medline search was conducted using "pediatric inflammatory bowel disease", "pediatric Crohn's disease", "linear growth", "pubertal growth", "bone health", and "vitamin D" as key words. Clinical trials, systematic reviews, and meta-analyses published between 2008 and 2013 were selected to produce this narrative review. Studies referring to earlier periods were also considered if the data was relevant to our review. Although current treatment strategies for CD that include anti-tumor necrosis factor-α therapy have been shown to improve patients' growth rate, linear growth deficiencies are still common. In pediatric CD patients, prolonged diagnostic delay, high initial activity index, and stricturing/penetrating type of behavior may cause growth deficiencies (in weight and height) and delayed puberty, with several studies reporting that these patients may not reach an optimal bone mass. Glucocorticoids and inflammation inhibit bone formation, though their impact on skeletal modeling remains unclear. Long-term control of active inflammation and an adequate intake of nutrients are both fundamental in promoting normal puberty. Recent evidence suggests that recombinant growth factor therapy is effective in improving short-term linear growth in selected patients, but is of limited benefit for ameliorating mucosal disease and reducing clinical disease activity. The authors conclude that an intense initial treatment (taking a "top-down" approach, with the early introduction of immunomodulatory treatment) may be justified to induce and maintain remission so that the growth of children with CD can catch up, ideally before puberty. Exclusive enteral nutrition has a key role in inducing remission and improving patients' nutritional status.

Key words: Bone health; Enteral nutrition; Growth; Height; Pediatric inflammatory bowel disease; Pediatric Crohn's disease; Linear growth; Pubertal growth; Vitamin D; Weight loss

Core tip: This review focuses on current evidence for managing growth issues in children diagnosed with Crohn's disease. Long-term control of active inflammation and an adequate intake of nutrients are both essential in promoting puberty. Exclusive enteral nutrition has a key role, as it induces disease remission and improves nutritional status. The early introduction of immunosuppressants or biologics may be justified in children to achieve disease remission and enable their growth to catch up, ideally before puberty. Recent evidence suggests that recombinant growth factor therapy is effective in improving short-term linear growth.

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INTRODUCTION

Crohn's disease (CD) is a global health concern and a condition that significantly affects patients' quality of life, as well as placing a heavy financial burden on the community[1]. CD is currently without a cure, and its incidence is rising not only in Western countries, but also in most developing countries. It manifests in childhood or adolescence in up to 25% of cases[2].

The microbial ecosystem colonizing the human bowel is influenced by diet, which prompts metabolic processes essential to bowel metabolism[3-7]. Genetic susceptibility, intestinal microbiota, lifestyle and environmental factors are amongst the potential mechanisms involved in the pathogenesis of inflammatory bowel diseases (IBD)[3]. Prolonged diagnostic delays, high initial activity indexes, and stricturing/penetrating behavior patterns may predict subsequent complications and the need for surgery, thus justifying a resort to early intensive therapy. The early introduction of immunomodulatory therapy favorably affects the course of IBD[8-12]. Growth failure and impaired nutritional status are seen in 65%-85% of children and adolescents diagnosed with CD, and 15%-40% of these patients continue to suffer from growth deficiency throughout the course of their disease[1,13]. Exclusive enteral nutrition has become a key treatment strategy for inducing disease remission in pediatric CD, offering the advantage of improving patients' nutritional status as well as enabling the mucosa to heal at much the same rate as is achievable with corticosteroids[1,14-17].

GROWTH ISSUES IN PEDIATRIC CROHN'S DISEASE

Growth deficiency can severely affect quality of life for children and adolescents with CD, and complicate their management[18-20]. It occurs in a significant proportion of patients (up to 85%), and may even precede any clinical evidence of bowel disease. Abraham *et al*[21] recently conducted a systematic review focusing on understanding the long-term risks of growth deficiency, disease reclassification and extension, hospitalization, cancer, and death among patients with childhood IBD[21] (Table 1): in 41 studies considered, concerning 3505 patients with CD, 2071 with ulcerative colitis (UC) and 461 with non-IBD colitis, growth failure was identified more often in CD (10%-56%) than in UC (0%-10%) or non-IBD controls. Growth improved after surgical resection in patients with CD[21].

Among IBD sufferers, male patients are more vulnerable to growth deficiencies than females of the same age because the male growth spurt in puberty is greater, occurs later, and lasts longer than in females[22]. Vasseur *et al*[23] examined a total of 261 pediatric patients with CD registered in the EPIMAD registry in northern France. At diagnosis, 25 children (9.5%) had a height more than 2 standard deviations below the norm, and the same applied to the weight of 70 children (27%), and the BMI of 84 children (32%). At maximal follow-up, 18 children (6.9%) had a growth deficiency and 40 (15%) suffered from malnutrition. Nutritional status was more severely impaired in children with stricturing disease. Growth and nutritional deficiency at diagnosis, young age, male gender, and extraintestinal manifestations at diagnosis were indicators of a poor prognosis. The authors concluded that young boys with substantial inflammatory manifestations of CD are at higher risk of subsequent growth failure, especially when their growth is already deficient at diagnosis[23].

Assessing growth in IBD patients of a developmental age is so important that it was included among the key points in the Paris classification of pediatric CD, which replaced the previous Montreal classification[24]. The following factors are implicit in the physiopathology of growth deficiency in pediatric IBD[22]: (1) chronic calorie insufficiency: these patients' malnutrition is due to a lower intake, protein malabsorption, abnormal intestinal losses, and anorexia correlating with the pathological picture [tumor necrosis factor-α (TNF-α) also has a direct influence at the hypothalamic level]. Inflammatory mediators trigger an increase in basal metabolism too, coinciding with a further deterioration in nutritional status; (2) direct cytokine effects: insulin-like growth factor (IGF)-1 is produced in the liver and is the principal mediator of the effects of growth hormone (GH). Patients suffering from CD have significantly reduced IGF-1 blood levels, irrespective of their GH levels. TNF-α and interleukin (IL)-6 also have a direct inhibitory effect on GH. Other pathways independent of IGF-1 inhibition by means of which the inflammatory cytokines inhibit the linear growth rate have recently been identified as well; (3) effects of chronic treatment with corticosteroids: these drugs induce a central suppression of GH production and reduce IGF-1 synthesis in the liver, as well as interfering with its peripheral receptor activity; (4) effects of IBD on endocrine growth mediators: delayed puberty gives rise to a sex hormone deficiency that may be involved in growth deficiencies; and (5) genetic factors: polymorphisms of NOD2/CARD15 and other already-identified genes appear to generate a cytokine pattern capable of contributing to pediatric IBD patients' growth deficiency; promoter regions of the gene coding for TNF-α and IL-6 also seem to be involved.

NUTRITIONAL CONCERNS IN PEDIATRIC CROHN'S DISEASE

Nutritional issues are often associated with CD, especially in pediatric cases, with underweight and stunting

Table 1 Summary of the main studies that were reviewed on nutritional concerns in pediatric Crohn's disease

Ref.	Type of study	Patients	Results	Conclusion
Vaisman *et al*[25], *Nutrition* 2006	Prospective cohort study	16 pts with CD; Age 19-57 yr Remission of disease (CDAI Activity Disease Index < 150); 2 groups (BMI 18.5 kg/m^2 as a cutoff point)	Subjects with lower BMIs tended to have less lean body mass (P = 0.006), less bone mineral density (P = 0.006), and lower resting energy expenditure (P = 0.003); No correlation between BMI and energy intake, although percentage of malabsorption negatively correlated with BMI (P = 0.07)	In the presence of similar energy intake, resting energy expenditure does not seem to contribute to lower BMI, although nutrient malabsorption is higher in malnourished patients with CD in remission; Malabsorption should be evaluated in patients with CD who fail to gain Wt during disease remission, to establish their extra caloric requirements
Gupta *et al*[28], *Inflamm Bowel Dis* 2013	Retrospective review	43 IBD pts (mean age 12.8 yr; range 5.1-17.4 yr) 67% M 33% F	Reductions in erythrocyte sedimentation rate (P < 0.0001) and C-reactive protein (P < 0.02), and increases in albumin (P < 0.03); Mean PCDAI score 26.9 at baseline and 10, 2 at follow-up (P < 0.0001); Induction of remission achieved in 65% and response in 87% at a mean follow-up of 2 mo (1-4 mo)	Novel protocol for enteral nutrition (80%-90% of patient's caloric needs) seems to be effective for the induction of remission in CD children; The protocol may result in improved EN acceptance and compliance and will be evaluated prospectively
Wiskin *et al*[29], *J Hum Nutr Diet* 2012	Prospective cohort study	46 IBD children	No children scored low risk with STAMP, STRONGkids or PNRS; 23 children scored low risk with PYMS; Good agreement between STAMP, STRONGkids, and PNRS (K > 0.6); Modest agreement between PYMS and the other scores (K = 0.3); No agreement between the risk tools and the degree of malnutrition based on anthropometric data (K < 0.1)	Relevance of nutrition screening tools for children with chronic disease is unclear; There is the potential to under recognize nutritional impairment (and therefore nutritional risk) in children with IBD
Valentini *et al*[30], *Nutrition* 2008	Prospective, controlled, multicentric study	94 pts with CD (CDAI 71 +/- 47) 61 F 33 M 50 UC (UCAI 3.1 +/- 1.5) 33 F 17 M 61 healthy control subjects 41 F 20 M from centers in Berlin (Germany), Vienna (Austria), and Bari (Italy) 47 well-nourished patients with IBD pair-matched to healthy controls by BMI, sex, and age	74% IBD patients were well-nourished according to the SGA, BMI, and serum albumin; Body composition analysis demonstrated a decrease in BCM in patients with CD (P = 0.021) and UC (P = 0.041) compared with controls; Handgrip strength correlated with BCM (r = 0.703, P = 0.001) and was decreased in patients with CD (P = 0.005) and UC (P = 0.001) compared with controls; Lower BMC in patients with moderately increased serum CRP levels compared with patients with normal levels	In CD and UC, selected micronutrient deficits and loss of BCM and muscle strength are frequent in remission and cannot be detected by standard malnutrition screening
Chan *et al*[31], *Am J Gastroenterol* 2013	Prospective cohort study	300724 participants (recruited into the European Prospective Investigation into Cancer and Nutrition study) 177 UC and 75 CD	No associations with the four higher categories of BMI compared with a normal BMI for UC (P trend = 0.36) or CD (P trend = 0.83); Lack of associations when BMI analyzed as a continuous or binary variable (BMI 18.5 kg/m^2 *vs* ⩾ 25 kg/m^2); Physical activity and total energy intake not associated with UC (P trends 0.79-0.18) or CD (P trends 0.42-0.11)	Obesity as measured by BMI not associated with the development of incident UC or CD; Alternative measures of obesity required to further investigate the role of obesity in the development of incident IBD
Werkstetter *et al*[32], *J Crohns Colitis* 2012	Prospective cohort study	39 IBD children in remission; 27 CD, 12 UC 24 M; 39 healthy age-sex-matched controls	IBD pts *vs* controls: Lower Z-scores for phase angle α [-0.72; 95%CI: (-1.10-0.34)] Lower grip strength [-1.02 (-1.58-0.47) Lesser number of steps per day [-1339 (-2760-83)] Shorter duration of physical activity [-0.44 h (-0.94-0.06)], particularly in F and patients with mild disease. Quality of life and energy intake did not differ between patients and controls	In spite of quiescent IBD, lean body mass and physical activity were reduced; Interventions to encourage physical activity may be beneficial in this lifelong disease

Gerasimidis *et al*[33] *Inflamm Bowel Dis* 2013	Prospective cohort study	184 new pediatric IBD Dg 139 one year follow-up IBD children 84 children treated with EEN	72% anemic at Dg; Anemic children with CD had shorter diagnosis delay, lower BMI, lower Dg delay ($P < 0.001$) and BMI Z-score, $P = 0.003$) than non-anemic patients; Extensive colitis associated with severe anemia in UC; After EEN, severe anemia decreased (32%-9%, $P < 0.001$) and hemoglobin concentration increased by 0.75 g/dL	Anemia is frequent at Dg and follow-up and should receive more attention from the clinical team; The focus should remain suppression of inflammatory process in active disease

Dg: Diagnosis; pt/pts: Patient/patients; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Unclassified IBD; rhGH: Recombinant growth factor; ESR: Erythrocyte sedimentation rate; BMC: Body cell mass; BMI: Body mass index; Ht: Height; Wt: Weight; BMD: Bone mineral density; DXA: Dual-energy X-ray absorptiometry; EEN: Exclusive enteral nutrition; GH: Growth hormone; (P)CDAI: (Pediatric) Crohn's disease activity index; PUCAI: Pediatric ulcerative colitis activity index; M: Male; F: Female.

commonly seen at presentation, as well as linear growth retardation and delayed puberty developing later on[1]. Undernutrition has been reported in 65%-75% of patients with CD[25] (Table 1), and recent weight loss is one of the triad of clinical manifestations of the disease. Although medical treatment can soon restore body weight, this is not reflected in concomitant changes in body composition. Children with CD have the features of nutritional cachexia, with normal fat stores but depleted lean mass. Poor bone health, delayed puberty, and growth failure are other possible features complicating their clinical management[26].

As growth impairment is mainly secondary to disease activity, all available pharmacological steps to induce remission in a given patient (depending on their disease phenotype) should have a positive effect on growth as well. Exclusive enteral nutrition has been used as a therapeutic approach to CD because it can improve patients' nutritional status and induce remission (mucosal healing) as quickly as corticosteroids[1,27]. Exclusive enteral nutrition has thus become a fundamental option at many centers treating pediatric CD[1]. A recent retrospective review by Gupta *et al*[28] (Table 1) assessed the efficacy of enteral nutrition (EN) in delivering 80%-90% of patients' calorie needs with a view to inducing remission in pediatric patients with CD. This approach allowed for patients to ingest the remainder of the calories they needed from a normal diet, and so it differs from the standard practice of providing EN to cover 100% of patients' calorie needs. The sample's mean Pediatric Crohn's Disease Activity Index score (PCDAI) at the baseline was 26.9 and it dropped to 10.2 at follow-up ($P = 0.0001$). Remission was induced in 65% of cases and response in 87% after a mean 2 mo of follow-up (1-4 mo). The authors concluded that this novel EN protocol seems to be effective in inducing remission in pediatric patients with CD, helping to increase their weight and improve their laboratory markers. This protocol may also make EN more readily acceptable to patients and improve their compliance[28].

There has recently been increasing interest in the use of nutrition risk assessment tools in children to identify those needing nutritional support[29] (Table 1). Four screening tools that are not disease-specific [the Screening tool for the assessment of malnutrition in pediatrics (STAMP), the screening tool for risk on nutritional status and growth (STRONGkids), the pediatric Yorkhill malnutrition score (PYMS), and the simple pediatric nutrition risk score (PNRS)] were applied by Wiskin *et al*[29] to 46 children with IBD. The degree of malnutrition was measured by anthropometry alone using the World Health Organization's International Classification of Diseases (ICD-10) criteria. There was a good agreement between STAMP, STRONGkids, and PNRS ($K > 0.6$), but only a modest agreement between PYMS and the other scores ($K = 0.3$), and no agreement between the risk tools and the degree of malnutrition based on anthropometric data ($K < 0.1$). The authors concluded that the relevance of nutrition screening tools for children with chronic intestinal disease is unclear, and there is a risk of their failing to recognize nutritional impairment (and consequent nutritional risk) in children with IBD[29].

A study by Vaisman *et al*[25] (Table 1) focused on identifying the relative contribution of factors causing malnutrition in a sample of 16 patients with CD in remission (age 19-57 years). Resting energy expenditure (REE) was studied by indirect calorimetry and body composition by dual-energy X-ray absorptiometry. Subjects with lower BMIs tended to have less lean body mass ($P = 0.006$), a lower bone mineral density ($P = 0.006$), and lower REE ($P = 0.003$). No correlation emerged between BMI and energy intake, although the percentage of malabsorption correlated negatively with BMI ($P = 0.07$). The authors concluded that nutrient malabsorption is more severe in malnourished patients with CD in remission, and consequently suggested that malabsorption should be assessed in CD patients who fail to gain weight while in remission in order to establish their extra calorie needs[25].

A prospective, controlled, multicentric study by Valentini *et al*[30] (Table 1) considered nutritional status (subjective global assessment [SGA], BMI, albumin, trace elements), body composition (bioelectrical impedance analysis, anthropometry), muscle strength, and quality of life in 94 patients with CD and 50 with UC, all in clinical remission, and 61 healthy controls. Most patients with IBD (74%) were well-nourished according to their SGA, BMI, and serum albumin levels, but body composition analysis demonstrated a lower body cell mass (BCM) in patients with CD ($P = 0.021$) and UC ($P = 0.041$) than in

controls. Handgrip strength correlated with BCM (P = 0.001) and was again lower in patients with CD (P = 0.005) and UC (P = 0.001) than in controls. These differences were seen even in patients classified as well-nourished. BCM was lower in patients with moderately increased serum C-reactive protein levels than in patients with normal levels. The authors concluded that selected micronutrient deficits and loss of BCM and muscle strength are frequent in CD and UC in remission, and go undetected in standard malnutrition screening[30].

Obesity is associated with a pro-inflammatory state that may be involved in the etiology of IBD. Chan *et al*[31] (Table 1) conducted the first prospective cohort study to identify any association between obesity and the onset of incident IBD in a sample of 300724 participants recruited for the European Prospective Investigation into Cancer and Nutrition study. At recruitment, anthropometric measurements were taken of patients' height and weight, and their physical activity and total energy intake were recorded using validated questionnaires. The cohort was monitored and 177 participants developed incident UC, while 75 developed incident CD. No associations emerged vis-à-vis UC or CD between the four higher BMI categories and a normal BMI level. Physical activity and total energy intake (factors influencing BMI) also revealed no association with UC or CD. The authors concluded that obesity, as measured by the BMI, is unassociated with the onset of incident UC or CD. Alternative obesity measures are needed to further clarify the role of obesity in the onset of incident IBD[31].

Physical activity is important for muscle and bone strength in growing children and may be limited in pediatric IBD patients, even when their disease is quiescent[32]. A recent study by Werkstetter *et al*[32] (Table 1) compared 39 IBD patients (27 CD, 12 UC) in remission (or with only mild disease activity) with 39 healthy age- and sex-matched controls. The patients had lower Z-scores for phase angle α and lower handgrip strength than controls. They tended to take fewer steps per day and engage in shorter periods of physical activity, particularly among females and patients with mild disease. The authors concluded that, even with quiescent disease, IBD patients have reduced levels of lean body mass and physical activity. Action to encourage them to engage in physical exercise may therefore be beneficial in this lifelong disease[32].

Low concentrations of plasma micronutrients are commonly reported in IBD patients, but may be difficult to interpret in the presence of an acute phase response, and other body store adequacy indices are needed. Anemia is a common extraintestinal manifestation in IBD children: these are primarily cases of iron-deficiency anemia, with anemia of chronic disease coming second[33]. A study by Gerasimidis *et al*[33] (Table 1) explored the epidemiology of anemia and associated factors in children with IBD at the time of their diagnosis, after 1 year, and during treatment with exclusive enteral nutrition (EEN). At diagnosis, 72% of the children were anemic. Children with CD who were anemic had a shorter diagnostic delay and a lower BMI than those who were not (P = 0.003). Extensive colitis was associated with severe anemia in UC. After EEN, the cases of severe anemia decreased (32%-9%; P = 0.001), and hemoglobin concentrations increased by 0.75 g/dL. The authors concluded that children with IBD are highly likely to have anemia at diagnosis, and that this matter should receive more attention during their follow-up, even though clinicians should focus on suppressing the inflammatory process in cases of active disease[33].

Deficiencies in the liposoluble vitamins A-D and E, and zinc are also possible features of IBD patients[34-36].

Vitamin D

Vitamin D is a key factor not only for its role in the mineralization of bone and teeth, but also because of its other metabolic functions and its protective role in immune-mediated diseases and allergies[37-39]. Vitamin D status is assessed by testing its metabolite 25-hydroxy-vitamin D (25-OH-D) in plasma or serum, which reflects the amount of vitamin D converted in the skin through sunlight exposure and ingested in the diet[37]. Poor vitamin D status may have detrimental consequences for the future health of a child, so an optimal vitamin D status is a crucial public health goal. Vitamin D levels are classed as severely deficient for levels < 37 nmol/L, insufficient for levels < 50 nmol/L, and suboptimal for levels of 50-75 nmol/L[37]. Exposure to sunlight is generally the most important source of vitamin D3, while the contribution of vitamin D from foods and supplements is fundamental in populations living at latitudes with limited hours of sunlight[37]. Obesity is a risk factor for vitamin D deficiency because a greater proportion of the body's vitamin D remains stored in adipose tissue[37].

Current national recommendations suggest a daily intake of 7.5 mcg of vitamin D[37]. Foods containing large amounts of vitamin D include oily fish and eggs. Vitamin D is produced endogenously in the skin by the photo-reduction of 7-dehydrocholesterol by ultraviolet light. Human exposure to sunlight is limited throughout their lives and some foods are fortified with vitamin D (*e.g.*, milk, some juices, breads, and cereals). Children with chronic diseases are consequently at risk of vitamin D deficiency. The Institute of Medicine recommends a vitamin D intake of 600 IU/d in individuals 1-70 years of age, plus 700-1300 mg/d of calcium (depending on age) to promote healthy skeletal growth[40]. Vitamin and mineral deficiencies have been described in patients with IBD and are attributed to gut mucosa inflammation and a reduced oral intake[34]. Cranney *et al*[41] conducted a systematic qualitative review of 167 eligible studies (112 randomized controlled trials (RCTs), 19 prospective cohort studies, 30 case-control, and 6 before-after studies). The largest body of evidence on vitamin D status and bone health concerned older adults, while few studies focused on infants, children, and adolescents. There was inconsistent evidence of an association between circulating 25(OH)D levels and bone mineral content in infants. In adoles-

cents, there was a fair amount of evidence for an association between 25(OH)D levels and changes in BMD. There was solid evidence of the use of foods fortified with vitamin D (11 RCTs) consistently increasing serum 25(OH)D in both young and older adults. In short, the studies generated fairly good evidence of an association between circulating 25(OH)D concentrations and some bone health outcomes (established rickets, PTH, falls, BMD). When compared with a placebo, vitamin D(3) (> 700 IU/d) with calcium supplementation reportedly had a small beneficial effect on BMD and reduced the risk of fractures and falls, although this benefit may be confined to specific subgroups[42]. A recent retrospective study performed by Alkhouri *et al*[34] in the US investigated the prevalence of vitamin and zinc deficiencies in 61 children (age 1-18 years) with newly-diagnosed IBD from 2006 to 2010 (80% with ileal inflammation) by comparison with a control group of 61 age- and sex-matched individuals. While none of the IBD patients had folate or vitamin B12 deficiency, 62% of them had vitamin D deficiency (*vs* 75% in the control group), 16% had vitamin A deficiency, 5% had vitamin E deficiency (*vs* 8% in the control group), and 40% had zinc deficiency (*vs* 19% in the control group). The authors concluded that vitamin B12 and folate deficiencies are rare in children with newly-diagnosed IBD in the United States, so there is no reason to support their routine monitoring. On the other hand, vitamin A and zinc deficiencies were statistically more prevalent among the IBD cases than in controls, so their levels should be assessed at the time of their diagnosis to enable enteral supplementation to be started[34]. Vitamin D deficiency was common in the population tested, so routine screening for this deficiency and supplementation are warranted. These results contrast with previous studies by Yakut *et al*[43] and Chowers *et al*[44].

To sum up on vitamin D, there is still no strong evidence in the literature of an association between circulating 25(OH)D concentrations and bone health outcomes, and an improvement in BMD in particular. Only partial benefits of administering vitamin D(3) (> 700 IU/d) and calcium supplementation have been seen in terms of BMD and a lower risk of fractures and falls, and only in specific subgroups[41]. Supplementing vitamin D in pediatric patients is nonetheless generally recommended when its levels are found to be depleted, given its action not only on bone metabolism but also in terms of immunomodulation[37,40].

BONE HEALTH AND PEDIATRIC CROHN'S DISEASE

Although current treatment strategies for CD that include therapy against TNF-α have been found to speed up growth, linear growth deficiencies persist even with optimized therapy[22]. Children with CD continue to suffer from short stature and slow growth, and several studies have indicated that children with IBD may fail to achieve optimal bone mass[45-47]. Children with CD have multiple risk factors for impaired bone accrual[48]. The skeleton is a highly dynamic tissue regulated by local, systemic, and environmental clues that modify osteoblastic (bone formation) and/or osteoclastic (bone reabsorption) activities. IBD affects bone regulation at all levels: environmentally through intestinal barrier breaks and/or a microbial composition in the gut; systemically with the circulation of gut immune cells and cytokines throughout the body; and locally by causing inflammation of extra-intestinal organs (such as the bone marrow)[49].

Bone formation and reabsorption are significantly involved in bone health and growth. In children with CD, both of these processes are impaired, so bone growth is ultimately suboptimal[49]. Factors contributing to this derangement are inflammation, delayed growth and puberty, lean mass deficiencies, and the use of glucocorticoids[50,51]. A recent study by Irwin *et al*[52] examined the effect of experimental IBD on bone health. Interleukin-10-deficient animals infected with *Helicobacter hepaticus* (*H. hepaticus*) were used as a murine model of colitis, and the molecular and histological properties of their bone and intestine were examined to identify the immunopathological consequences of colitis in mice. Six weeks after they were infected, male (but not female) mice revealed significant trabecular bone loss in the distal femur and vertebrae. The authors concluded that the severity of *H. hepaticus*-induced colitis and the associated bone loss are gender-related, possibly as a result of gender-specific effects on *H. hepaticus* colonization in the mouse gastrointestinal tract and the consequent immunopathological responses[52].

A prospective study by Kim *et al*[45] (Table 2) aimed to examine the risk factors and extent of bone mass reduction and to analyze the impact of IBD developing early, before bone mass has peaked (*i.e.*, before the maximal bone mineral density has been reached during development). Bone mineral density (BMD) was assessed in the lumbar spine and hip bone in 44 IBD patients (21 of them < 30 years old). Younger patients had a significantly more severe bone mass reduction in the lumbar spine than patients aged > 30 years (multivariate analysis showed a hazard ratio of 3.96, $P = 0.06$)[45]. On the other hand, a recent prospective cohort study by Tsampalieros *et al*[53] (Table 2) suggested that younger age provides a window of opportunity for skeletal recovery. The aim of their study was to examine changes in BMD and cortical structure after CD had been diagnosed, and to identify associations with growth, glucocorticoids, and disease activity. The authors concluded that CD was associated with a persistently low trabecular BMD, although younger participants showed a greater potential for recovery. A greater linear growth was associated with a greater recovery of cortical dimensions, especially among participants with less glucocorticoid exposure and inflammation. So, although glucocorticoids and inflammation inhibit bone formation, their impact on skeletal modeling is still not clear[53].

Another longitudinal study performed by Schmidt *et al*[51] (Table 2) on a total of 144 patients with IBD (including 83 with UC and 45 with CD) concluded that IBD

Table 2 Summary of the main studies that were reviewed on growth issues and bone health in pediatric Crohn's disease

Ref.	Type of study	Patients	Results	Conclusion
Abraham *et al*[21] *J Clin Gastroenterol* 2012	Systematic review	3505 CD, 2071 UC, and 461 IBD-U (age at onset < 18 yr)	Growth failure was reported in CD (10% and 56%) more often than UC (0%-10%) or non-IBD controls; Improvements in growth occurred after surgical resection in CD pts; Increase in disease reclassification over time from UC and IBD-U Dg to CD; CD pts had higher number of hospitalizations and hospital days per year *vs* UC pts in most studies; The reported surgery rates in CD ranged between 10% and 72%; the colectomy rates in UC ranged between 0% and 50%	Childhood-onset IBD pts had growth failure reported in pts with CD more often than those with UC, and had a reclassification of disease type to CD over time; Higher rates of surgery and hospitalizations were found with CD than with UC
Kim *et al*[45] *Clin Endosc* 2013	Prospective cohort study	44 IBD (21 aged < 30 yr; 23 aged > 30 yr)	Significant bone mass reduction at the LS in IBD patients aged < 30 yr *vs* patients aged > 30 yr (BMD $P < 0.01$; T-score $P < 0.01$; Z-score $P < 0.01$); Multivariate analysis: risk factor of bone mass reduction for patients < 30 yr → HR = 3.96, $P = 0.06$	Bone mass reduction is more severe in patients diagnosed with IBD before the age of 30 yr
Schmidt *et al*[51] *J Pediatr Gastroenterol Nutr* 2012	Longitudinal cohort study	144 IBD pts 83 UC, 45 CD	Children with UC and CD had significantly lower mean BMD Z-scores for the LS at baseline and after 2 yr; The reduction in BMD was equally pronounced in patients with UC and CD; Neither group improved their Z-score during the follow-up period; Significantly lower mean BMD Z-scores for the LS were found at baseline in M ($P < 0.001$), but not in F; Lowest BMD values in the group of patients ages 17 to 19 yr in M and in F	The entire group of pediatric patients with IBD showed permanent decreases in their BMD Z-scores for the LS; however, afflicted children have the potential to improve their BMD by the time they reach early adulthood
Tsampalieros *et al*[53] *J Clin Endocrinol Metab* 2013	Prospective cohort study	CD (age 5-21)	Disease activity improved over the study interval ($P < 0.001$); Trabecular BMD-Z improved over the first 6 mo; Increases associated with improved disease activity ($P < 0.001$), younger age ($P = 0.005$), and increases in vitamin D levels ($P = 0.02$); Greater increases in tibia length associated with greater increases in cortical area-Z ($P < 0.001$); Greater glucocorticoid doses and disease activity significantly associated with failure to accrue cortical area, and more pronounced with greater linear growth (interaction $P < 0.05$); Mean ± SD trabecular BMD and cortical area Z-scores significantly reduced at the final visit	CD was associated with persistent deficits in trabecular BMD; Younger participants demonstrated greater potential for recovery; Greater linear growth associated with greater recovery of cortical dimensions, especially among participants with lesser glucocorticoid exposure and inflammation; Younger age and concurrent growth provide a window of opportunity for skeletal recovery
Malik *et al*[54] *J Crohns Colitis* 2012	Prospective cohort study	36 children with CD (Male 22)	28 (78%) CD children treated with adalimumab went into remission; Overall 42% of children showed catch-up growth, which was more likely in: Pts who achieved remission ($P = 0.007$); Pts who were on immunosuppression ($P = 0.03$); Pts whose indication for adalimumab was an allergic reaction to infliximab ($P = 0.02$); Pts who were on prednisolone when starting adalimumab, ($P = 0.04$)	Clinical response to adalimumab is associated with an improvement in linear growth in a proportion of children with CD; Improved growth is more likely in patients entering remission and on immunosuppression but is not solely due to a steroid sparing effect
Malik *et al*[55] *Arch Dis Child* 2012	Retrospective cohort study	116 CD children; 68 M; Mean age at diagnosis 10.8 yr (range 4.9-15.5); Mean age at maximum follow-up of 15.4 yr (9.4-19.3)	At T0, mean height SD score was -0.5 (-3.3-2.6) compared to a mid-parental mean height SD score of 0.2 (-2.0-1.4) ($P = 0.002$); At T1, T2, T3, and maximum follow-up, mean height SD score was -0.6 (-4.8-7.8), -0.6 (-2.9-2.2), -0.7 (-3.6- 2.5) and -0.5 (-3.5-2.9), respectively; Mean Ht velocity SDS at T1, T2, T3 and maximum follow-up was -1.4 (-7.4-7.4), -0.6 (-7.5-6.1), -0.1 (-6.6 -7.6) and 0.6 (-4.8-7.8), respectively ($P < 0.05$)	In final models: Mean Ht velocity SDS was associated negatively with the use of prednisolone ($P = 0.0001$), azathioprine ($P = 0.0001$), methotrexate ($P = 0.0001$), and weight SDS (WtSDS) $P = 0.0001$); Mean Ht velocity SDS was associated positively with age ($P = 0.0001$) and Wt SDS ($P = 0.01$); ΔHt SDS was associated negatively with use of prednisolone ($P < 0.02$)

Laakso *et al*[56] *Calcif Tissue Int* 2012	Cross-sectional Cohort Study	80 IBD pts (median age 14.9 yr, range 5-20), median disease duration 3.4 yr; 51 UC, 26 CD, and 3 IBD-U	IBD pts had lower bone age-adjusted LS and whole-body areal BMD ($P < 0.001$ for both) and whole-body composition adjusted for Ht ($P = 0.02$) than controls; Lean mass and fat mass Z-scores did not differ between the groups, but IBD patients had lower whole-body composition relative to muscle mass ($P = 0.006$); Vitamin D deficiency in 48%, despite vitamin D supplementation; In IBD cumulative weight-adjusted prednisolone dose > 150 mg/kg for the preceding 3 yr increased the risk for low whole-body areal BMD (OR = 5.5, 95 %CI: 1.3-23.3, $P = 0.02$). Vertebral fractures found in 11% of patients and in 3% of controls ($P = 0.02$)	IBD in childhood was associated with low areal BMD and reduced bone mass accrual relative to muscle mass; The risk for subclinical vertebral fractures may be increased; Careful follow-up and active preventive measures are needed
Ahmed *et al*[60] *J Pediatr Gastroenterol Nutr* 2004	Prospective cohort study	47 CD and 26 UC (median age of 13.5 yr - range, 5.5-18.2 yr)	Pts with CD were shorter than those with UC ($P < 0.05$); Median ppBone Area for LS and total body for the whole group was 85% and 81%, respectively; ppBone Area at both sites was directly related to height SDS and BMI SDS ($r > 0.5$; $P < 0.005$); Median BMD SDS for LS and total body was -1.6 and -0.9, respectively; Median ppBMC for LS and total bone was 98% and 101%, respectively; ppBMC showed no relationship to ppBone Area ($r = 0.1$, NS); Children with osteopenia 22% after adjustment for bone area	Children with IBD often have small bones for age because they have growth retardation; When DXA data are interpreted with adjustment for bone size, most children have adequate bone mass; Correct interpretation of DXA is important for identifying children who may be at a real risk of osteoporosis
Burnham *et al*[61] *J Bone Miner Res* 2004	Prospective cohort study	104 children and young adults with CD 233 healthy controls (age 4-26)	CD pts had significantly lower Ht Z-score, BMI Z-score, and lean mass relative to Ht compared with controls (all $P < 0.0001$); After adjustment for group differences in age, Ht, and race, the ratio of BMC in CD relative to controls was significantly reduced in M (0.86; 95%CI: 0.83-0.94) and F (0.91; 95%CI: 0.85-0.98) with CD; Adjustment for pubertal maturation did not alter the estimate; addition of lean mass to the model eliminated the bone deficit; Steroid exposure was associated with short stature but not bone deficits	Importance of considering differences in body size and composition when interpreting DXA data in children with chronic inflammatory conditions; Association between deficits in muscle mass and bone in pediatric CD
Boot *et al*[62] *Gut* 1998	Prospective cohort study	55 pts (34 M 21 F, age range 4-18 yr) 22 CD, 33 UC	Mean SDS of LS BMD and total body BMD were significantly lower than normal (-0.75 and -0.95, both $P < 0.001$); Height SDS and BMI SDS were decreased. The decrease in BMD SDS could not be explained by delay in bone maturation; The cumulative dose of prednisolone correlated negatively with LS BMD SDS ($r = -0.32$, $P < 0.02$); BMI SDS correlated positively with total body BMD SDS ($r = 0.36$, $P < 0.02$); CD pts had significantly lower LS and total BMD SDS than UC pts, even after adjustment for cumulative dose of prednisolone; In the longitudinal data cumulative dose of prednisolone between the measurements correlated negatively with the change in LS and total BMD SDS; Lean tissue mass measured by dual X-ray absorptiometry had a strong correlation with lean body mass measured by bioelectrical impedance analysis ($r = 0.98$)	IBD children have a decreased BMD; CD children have a higher risk of developing osteopenia than UC children; Corticosteroid therapy and nutritional status are important determinants of BMD in IBD pts

Dg: Diagnosis; pt/pts: Patient/patients; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Unclassified IBD; rhGH: Recombinant growth factor; ESR: Erythrocyte sedimentation rate; BMC: Body cell mass; BMI: Body mass index; Ht: Height; Wt: Weight; BMD: Bone mineral density; DXA: Dual-energy X-ray absorptiometry; EEN: Exclusive enteral nutrition; GH: Growth hormone; (P)CDAI: (Pediatric) Crohn's disease activity index; PUCAI: Pediatric ulcerative colitis activity index; M: Male; F: Female.

children have the potential to improve their BMD by the time they reach early adulthood. Children with UC and CD had significantly lower mean BMD Z-scores for the lumbar spine (LS), both at the baseline and after 2 years. Sub-analyses of the different age groups at the baseline found the lowest BMD values for patients aged 17 to 19 years, be they boys or girls; at follow-up, these patients' BMD had significantly improved, however[51].

A recent study by Malik *et al*[54] (Table 2) assessed the frequency of short stature and poor growth, and how they correlated with the course of the disease and the therapy administered in children with CD. The anthropometric and treatment details regarding 116 children showed that mean height SDS was negatively associated with the use of prednisolone ($P = 0.0001$), azathioprine ($P = 0.0001$), or methotrexate ($P = 0.0001$) and with weight SDS (Wt SDS) ($P = 0.0001$)[54]. Another study by Malik *et al*[55] (Table 2) focused on growth and disease activity over 12 mo in 36 children with CD who started taking adalimumab. Disease remission was achieved in 78% of these cases, and an overall 42% of the children caught up in terms of their growth. This was more likely to happen for those in remission, taking immunosuppressants, and those starting adalimumab therapy due to an allergic reaction to infliximab. An increase in growth rate was also seen in 15 children who were on prednisolone therapy when they started taking adalimumab. The authors concluded that clinical response to adalimumab therapy is associated with an improvement in linear growth in some children with CD, and that this is more likely for patients entering remission and on immunosuppression, although the effect is not due to a steroid-sparing effect alone[55].

Another recent cross-sectional cohort study by Laakso *et al*[56](Table 2) compared the skeletal characteristics of 80 children and adolescents suffering from IBD with 80 healthy controls matched for age and gender. The IBD patients had a lower bone age (BA)-adjusted lumbar spine, total body bone area BMD ($P < 0.001$ for both), and whole-body bone mineral content (BMC) than controls, after adjusting for height ($P = 0.02$). Lean mass and fat mass z-scores did not differ between the groups, but IBD patients had a lower whole-body BMC relative to muscle mass ($P = 0.006$). Despite 48% of the IBD patients receiving vitamin D supplementation, deficiencies of this vitamin were common. In the IBD group, a cumulative weight-adjusted prednisolone dose > 150 mg/kg for the preceding 3 years increased the risk of low whole-body aBMD ($P = 0.02$). Vertebral fractures (VFs) were found in 11% of patients and 3% of controls ($P = 0.02$). The authors concluded that IBD in childhood is associated with a low aBMD and reduced bone mass accrual relative to muscle mass; the risk of subclinical VFs may increase. These observations warrant careful follow-up and active preventive measures[56].

There is a well-established relationship between the long-term use of glucocorticoids for any disease indication and a higher risk of osteoporosis and fractures[57-59], but the relationship between CD or UC and bone loss remains controversial. Inability to achieve peak bone mass when the disease starts in childhood, malnutrition, immobilization, low BMI, smoking, and hypogonadism may all have a part to play in the pathogenesis of bone loss. Although evidence is sparse on the topic of bone health in children and adolescents with IBD, most authors recommend bone health screening, monitoring growth parameters and pubertal development, checking vitamin D status and vitamin D and calcium intake, and prescribing exercise and nutritional support[30]. Bone health status should be assessed systematically in patients treated for more than 6 mo, particularly during puberty[50].

Assessing BMD with dual energy X-ray absorptiometry (DXA) generally involves a comparison with age- and gender-matched reference ranges, and such studies show a high prevalence of osteopenia in children with IBD[60]. A recent study by Ahmed *et al*[60] (Table 2) aimed to compare the prevalence of osteopenia using two interpretation methods, one adjusted for age and gender, the other adjusted for bone size and gender. Forty-seven patients with CD and 26 with UC were considered, and the former were found shorter than the latter (median height, SDS, - 0.9 *vs* 0, $P < 0.05$). The authors concluded that children with IBD often have small bones for their age because they have a growth deficiency. When DXA data were interpreted after adjusting for bone size, most of the children were found to have an adequate bone mass. It is therefore important to interpret DXA findings correctly to identify children who may be at real risk of osteoporosis[60].

A study by Burnham *et al*[61] (Table 2) was designed to assess BMC relative to growth, body composition, and maturation in CD cases compared with controls. Whole-body BMC and lean mass were assessed by DXA in 104 CD subjects and 233 healthy controls. CD was associated with significant deficits in BMC and lean mass, relative to height. Individuals with CD had significantly lower z-scores for height and BMI, and a lower lean mass relative to height than controls ($P < 0.0001$). After adjusting for group differences in age, height, and race, males and females with CD had a significantly lower BMC than controls. Steroid exposure was associated with short stature but not with bone deficits. This study pointed to the importance of considering differences in body size and composition when interpreting DXA data in children with chronic inflammatory conditions, as well as showing an association between deficits in muscle mass and bone in pediatric CD[61].

A study by Boot *et al*[62] (Table 2) assessed BMD, nutritional status, and determinants of BMD in 55 children with IBD (34 boys and 21 girls, age range 4-18 years; 22 years with CD, 33 years with UC). The mean SDS for lumbar spine BMD and total body BMD were significantly lower than normal (both $P < 0.001$). The SDS for height and BMI were low as well. The decrease in BMD SDS could not be explained by any delay in bone maturation. The cumulative dose of prednisolone correlated negatively with lumbar spine BMD SDS ($P < 0.02$). Patients with CD had significantly lower lumbar spine and total body BMD SDS than patients with UC, even after adjusting for the cumulative dose of prednisolone. The authors concluded that: children with IBD have a reduced BMD; children with CD are at higher risk of osteopenia than children with UC; and corticosteroid therapy and nutritional status are important determinants of BMD in these patients[62].

A very interesting report from Whitten *et al*[63] sup-

ported the role of enteral nutrition in improving bone metabolism. The authors enrolled 23 children with newly-diagnosed CD and 20 controls. Children with CD were treated for 8 weeks with EEN, and inflammatory markers, nutritional markers (height, weight), bone markers [C-terminal telopeptides of Type-1 collagen (CTX), and bone-specific alkaline phosphatase (BAP)] were measured before and after the treatment. At diagnosis, children with CD had higher serum CTX than controls ($P = 0.0003$). After the period of EEN, their CTX levels fell significantly ($P = 0.002$), and their serum BAP levels ($P = 0.07$) increased significantly ($P = 0.02$), both normalizing to control levels. This evidence indicates that, as well as reducing inflammation, decreasing disease activity, and improving nutrition in children with newly-diagnosed CD, EEN therapy also normalizes serum markers of bone turnover, suggesting an improvement in bone health[63].

To sum up, although current therapy for CD is associated with a better growth rate for the first few years, a substantial proportion of children with CD remain short. Depending on the population considered, the prevalence of osteoporosis has been variably reported to range from 12% to 42% in patients with IBD[13]. While prospective studies suggest sustained bone loss at both trabecular and cortical sites in long-term glucocorticoid users with IBD[57], a decrease in bone mass is also seen in patients with active CD not using glucocorticoids[49,50]. Be that as it may, it is strongly recommended that excessively long periods of corticosteroid therapy be scrupulously avoided, particularly for patients of developmental age, and enteral nutrition should be used (whenever possible) as an alternative front-line therapy because it helps to contain the need for corticosteroids and thus limits their unwanted effects on growth, as well as cosmetic issues (which are very important in adolescence)[22]. Data on vertebral fractures are scarce and there is no agreement about the risk of non-vertebral fractures in patients with CD, though it has been suggested that patients with IBD may carry a 60% higher risk of non-vertebral fractures. The main question is whether all patients with CD should be treated with bone-protecting agents on the assumption that they could all potentially develop osteoporosis, or whether these agents should be used only in patients clearly at risk of osteoporosis and fractures (providing such patients can be identified)[49].

PUBERTY-RELATED ISSUES IN PEDIATRIC CROHN'S DISEASE

Many nutritional, inflammatory, immunological, and endocrine factors affecting patients suffering from IBD and influencing their growth also have an important impact on the initiation and progression of puberty. The onset of IBD before puberty is frequently associated with an underdeveloped stature and weight, and with patients having a significantly slower growth rate and lower final height by comparison with the parental target. This is more evident in children with CD than in cases of UC[64,65]. Other correlations include delayed puberty and menarche, an extended duration of the pubertal phase, and secondary amenorrhea[64]. Potential causes of late puberty in patients developing IBD in pre-pubertal and pubertal age include[64]: (1) malnutrition: this correlates mainly with a delay in menarche and sexual maturity. A link has been suggested between late puberty and reduced fat mass, which is normally rich in the aromatases that induce the conversion of androgens into estrogens and the consequent active production of female hormones; and (2) interactions between proinflammatory cytokines and the endocrine system: endocrine functions seem to be disrupted in IBD patients, also due to a direct effect of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, on hormonal feedback mechanisms.

A recent retrospective study by Mason *et al*[66] (Table 3) aimed to ascertain the impact of CD and UC on the pubertal growth spurt. Pubertal growth was assessed by calculating peak height velocity (HV) SDS (PHV SDS), height SDS at diagnosis, height SDS at PHV, and age at PHV in patients with CD (30 boys, 11 girls) and UC (14 boys and 12 girls). Systemic markers of disease activity were also recorded. Altered pubertal growth parameters were apparent in the CD cases by comparison with the normal population, particularly in boys. In the group as a whole, age (PHV) showed an association with erythrocyte sedimentation rate ($r = 0.4$; $P = 0.005$) and an inverse association with BMI ($r = 0.4$; $P = 0.001$)[66].

MANAGEMENT OF GROWTH AND PUBERTAL ISSUES IN PEDIATRIC CROHN'S DISEASE

Healthy children grow at an annual rate of 4-6 cm up until puberty, when their rate of growth doubles for over a year[22]. A declining trend in growth chart percentiles for height and weight arouses the suspicion of growth deficiency vis-à-vis a child's targets for gender and age[22]. An early diagnosis of CD is fundamentally important, but the early signs of IBD vary and can easily go unnoticed, meaning that a statural growth deficiency and concomitant late puberty quite often precede the intestinal manifestations of the disease[22]. It is essential to monitor patients' growth, taking their initial height (as measured before the onset of IBD) for reference and routinely reassessing patients as their disease evolves in order to fully appreciate its impact on their growth[22]. Monitoring patients' growth rate is also important to see how they are responding to therapy over time[22]. Precise serial checkups should always include an assessment of patients' pubertal development, which should be correlated with their statural growth. If any discrepancies come to light, action can be taken without delay: radiology is used to establish patients' skeletal age and thus identify their residual potential for growth[22]. On average, it takes about 12 mo to see any response to treatment in terms of linear growth or pubertal development, so the intervals

Table 3 Summary of the main studies that were reviewed on management of growth and pubertal issues in pediatric Crohn's disease

Ref.	Type of study	Patients	Results	Conclusion
Mason *et al*[66] *Horm Res Paediatr* 2011	Retrospective cohort study	IBD adolescents 41 with CD, 30 M 11 F 26 with UC, 14 M 12 F	Altered parameters of pubertal growth observed in the CD groups compared to the normal population: In the CD M group, median Ht at Dg was -0.56 (P = 0.001) and median age at peak Ht velocity was 14.45 yr (P = 0.004) In the CD F group, median Ht at Dg was -1.14 (P = 0.007) and Ht at peak Ht velocity was -0.79 (P = 0.039). Individually, 8/30 CD M cases had one or more parameter affected: In the whole group, age at peak Ht velocity showed an association with ESR (r = 0.4; P = 0.005) and an inverse association with BMI (r = 0.4; P = 0.001)	Disorders of pubertal growth are more likely to occur in CD (particularly M)
Tietjen *et al*[69] *Turk J Gastroenterol* 2009	Prospective cohort study	40 pts with CD 26 M, 14 F mean age 16,7 yr (median: 17 yr, range: 4-29 yr)	Urinary GH levels were found as normal in CD; Corticosteroid therapy did not appear to be the most responsible factor for growth failure in CD	Growth failure in patients with CD is not caused by GH deficiency; A high PCDAI score has an important impact on impaired growth in children and adolescents with CD
Wong *et al*[70] *J Pediatr Endocrinol Metab* 2007	Retrospective data analysis	7 pts with CD 5 M	Median chronological age and median difference between chronological age and bone age was 15.9 yr (range, 13.0-17.9 yr) and 1.7 yr (-0.7-3.3 yr), respectively; Median dose of rhGH at T+0 was 0.23 mg/wk (0.15-0.31); Pubertal status remained unchanged in 6/7 patients; Median albumin and C-reactive protein were similar at T+0 and T+6; Median height SDS at T+0, T+6 and T+12 was -2.2 (-4.0 to -1.5), -1.9 (-4.1 to -0.8), -1.9 (-4.1 to -0.7), respectively (NS). Median Ht velocity SDS at T+0 and T+6 was -2.5 (-4.8-1.4) and -0.9 (-5.3 to 3.4), respectively (NS); Positive correlation between percentage change in Ht velocity SDS at T+6 and dose of rhGH at T+0 (r = 0.8, P = 0.03)	Introduction of rhGH therapy was associated with a cessation in the deterioration in linear growth; An improvement in Ht SDS was not observed over the period of the study
Wong *et al*[71] *Clin Endocrinol* (Oxf) 2011	Randomized controlled trial in 2 tertiary Children's Hospitals	22 children with IBD 21 with CD	Median Ht velocity increased from 4.5 (range, 0.6-8.9) at baseline to 10.8 (6.1-15) cm/year at 6 mo (P = 0.003) in the rhGH group, whereas in the Ctrl group, it was 3.8 (1.4-6.7) and 3.5 cm/yr (2-9.6), respectively (P = 0.58); Median percentage increase in Ht velocity after 6 mo in the rhGH group was 140% (16.7%-916.7%) compared with 17.4% (-42.1%-97.7%) in the Ctrl group (P < 0.001). No significant differences in disease activity and proinflammatory cytokines at baseline and 6 mo in both groups	rhGH can improve short-term linear growth in children with CD; The clinical efficacy of this therapy needs to be further studied in longer-term studies of growth, glucose homeostasis, and disease status

Dg: Diagnosis; pt/pts: Patient/patients; CD: Crohn's disease; UC: Ulcerative Colitis; IBD-U: Unclassified IBD; rhGH: Recombinant growth factor; ESR: Erythrocyte sedimentation rate; BMC: Body cell mass; BMI: Body mass index; Ht: Height; Wt: Weight; BMD: Bone mineral density; DXA: Dual-energy X-ray absorptiometry; EEN: Exclusive enteral nutrition; GH: Growth hormone; (P)CDAI: (Pediatric) Crohn's disease activity index; PUCAI: Pediatric ulcerative colitis activity index; M: Male; F: Female; NS: Not significant.

between follow-up assessments should never be less than six months[22].

To prevent and manage growth deficiencies in pediatric IBD patients, we must first establish the most appropriate nutritional, pharmacological, and surgical treatment for their underlying disease: managing their chronic inflammatory status and providing adequate nutrition are two synergically interacting aspects of the same approach[22]. Ensuring long-term control of active inflammation and administering an adequate intake of nutrients are both fundamental to promoting normal puberty[64]. Controlled clinical trials have documented a significant correlation between enteral nutrition, a reduced mucosal production of cytokines, and endoscopic healing. Enteral nutrition is potentially capable of inducing remission and achieving a nutritional recovery. Trials have also established that the effect of exclusively enteral nutrition on the inflammatory picture is influenced by factors such as the disease being localized in the small intestine or of recent onset, whereas the age factor appears to be less influential[22]. Immunomodulators other than corticosteroids used for pediatric IBD include the thiopurines (azathioprine and 6-mercaptopurine), which are used to maintain remission and have no demonstrated side-effects on growth, and biologics (infliximab and adalimumab), which potentially improve growth velocity by inducing and maintaining disease remission. Artificially-inducing puberty with the aid of estrogens and testosterone carries the risk of causing early growth cartilage calcification, giving rise to statural deficiencies[64].

Role of treatment with recombinant growth factor for pediatric Crohn's disease

Current treatment strategies for CD that include therapy against TNF-α have been found to improve growth velocity, but linear growth deficiencies persist even with optimized therapy[67]. Through complex mechanisms that include reducing IGF-1 levels and inducing systemic and hepatic GH resistance, cytokines such as TNF-α and IL-6 - which are commonly elevated in active CD - are important mediators of linear growth delay[68]. The potential for linear growth impairment as a complication of chronic intestinal inflammation is unique to pediatric CD patient populations[67]. IGF-I, produced by the liver in response to GH stimulation, is the key mediator of GH effects on the growth plate of bones. There is a well-known association between impaired growth in children with CD and low IGF-I levels. Early studies emphasized the role of malnutrition in suppressing IGF-I production. The direct, growth-inhibiting effects of pro-inflammatory cytokines have been increasingly recognized and explored. The role of non-cytokine factors (such as lipopolysaccharides) and their potential for negatively influencing the growth axis have also been investigated[67]. Recent evidence suggests that recombinant growth factor (rhGH) therapy is effective in improving short-term linear growth in selected patients[2], but is of limited benefit as a therapy for improving mucosal disease or reducing clinical disease activity[67]. A clinical analysis was performed by Tietjen *et al*[69] (Table 3) on 40 children, adolescents, and young adults with CD to see whether their growth failure was caused by impaired GH secretion. To assess growth hormone excretion, the authors measured urinary growth hormone with an in vitro immunoradiometric assay in three morning urine samples. They found normal urinary growth hormone levels in CD, concluding that growth failure in patients with CD is not caused by GH deficiency. Corticosteroid therapy did not appear to be the main culprit responsible for growth failure in CD either[69]. A retrospective data analysis was conducted by Wong *et al*[70] (Table 3) on 7 patients with CD treated with rhGH, after which the deterioration in their linear growth came to a stop, but no improvement in their height SDS was observed during the study period[70]. Another randomized controlled trial at two tertiary children's hospitals on 22 children with IBD (21 being cases of CD)[71] (Table 3) investigated the effects of rhGH on HV and glucose homeostasis over a 6-mo period. The median HV increased from 4.5 (range 0.6-8.9) at the baseline to 10.8 (6.1-15) cm/year at 6 mo (P = 0.003) in the rhGH group, while in the control group it was 3.8 (1.4-6.7) and 3.5 cm/year (2.0-9.6), respectively (P = 0.58). The median percentage increase in HV over 6 mo was 140% (16.7-916.7) in the rhGH group and 17.4% (42.1-97.7) in the control group (P < 0.001). There were no significant differences in disease activity or pro-inflammatory cytokines at the baseline or after 6 mo in either group, and the change in bone age for chronological age was also similar in the two groups[71]. This was the first randomized controlled trial on rhGH in children with IBD and growth retardation, and it showed - albeit over a brief period of 6 mo - that a dose of 0.067 mg/kg per day of rhGH improves linear growth. The authors also emphasized the continuing need to optimize the child's disease status (*i.e.*, to induce and maintain remission of IBD activity), as they found a greater growth response to rhGH in patients in biochemical remission. In short, although these data provide evidence of the efficacy of rhGH treatment in terms of height velocity over a short- to medium-term follow-up, patients treated with GH experienced no significant improvements in disease activity and pro-inflammatory cytokines by comparison with controls; and long-term follow-up data are lacking. In conclusion, based on currently-available evidence, the efficacy of rhGH in treating growth failure associated with CD is still unclear, and future studies should explore the use of higher doses of rhGH in CD[70].

CONCLUSION

Despite current treatment strategies for CD including anti-TNF-α medication, short stature and slow growth are still encountered in children with CD. Several studies have shown that children with IBD may not achieve optimal bone mass[25], and those with CD have multiple risk factors for impaired bone accrual[22]. A declining trend in growth chart percentiles for height and weight with respect to a patient's targets for gender and age should arouse the suspicion of a growth deficiency[22]. An early diagnosis is fundamentally important, but signs of the onset of IBD vary and can easily go unnoticed, meaning that statural growth deficiencies and concomitant late puberty quite often precede the intestinal manifestations of the disease[64].

Nutritional concerns are common in pediatric CD patients, who are often underweight at presentation[1]. Undernutrition has been reported in up to 65%-75% of such patients[25], and a low dietary intake due to poor appetite and aversion to food is a major cause of undernutrition in pediatric IBD, though the systemic release of proinflammatory cytokines also contributes significantly[26]. Although medical treatment can quickly restore body weight, this does not reflect concomitant changes in patients' body composition, which is characterized by normal fat stores but depleted lean mass. Poor bone health, delayed puberty, and growth failure may also complicate these patients' clinical management[26]. Vitamin and mineral deficiencies have been described in patients with IBD and are attributed to mucosal inflammation in the gut and a low oral intake. Although 25(OH) vitamin D levels have yet to be convincingly demonstrated to correlate with BMD[56], poor vitamin D status may have detrimental consequences for any child's future health, so an optimal vitamin D status still represents a crucial public health goal[37]. Corticosteroid therapy and nutritional status are important determinants of BMD in CD patients[49].

It is indispensable to monitor CD patients' growth, taking their initial height as a reference and routinely reassessing them as their disease evolves in order to fully appreciate its impact on their growth[22]. Monitoring patients' growth rate is also essential to enable their response to therapy to be assessed over time[22]. Precise serial check-ups should always include an assessment of patients' pubertal development, which should be correlated with their statural growth, so that action can be taken without delay in the event of any discrepancies coming to light. A radiological examination of patients' skeletal age enables their residual potential for growth to be identified[22]. Recent evidence suggests that rhGH therapy is effective in improving short-term linear growth for a selected group of CD patients, but is of limited benefit as a therapy for improving mucosal disease and reducing its clinical activity[70]. Exclusive enteral nutrition is a potentially effective option for treating CD because it can improve patients' nutritional state as well as inducing disease remission (mucosal healing) just as quickly as corticosteroids[1,27].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Biological therapy for ulcerative colitis: An update

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Abstract

Of the diverse biological agents used for patients with ulcerative colitis, the anti-tumor necrosis factor-α agents infliximab and adalimumab have been used in large-scale clinical trials and are currently widely used in the treatment of inflammatory bowel disease patients. Recent studies have indicated that golimumab, oral tofacitinib and vedolizumab reportedly achieved good clinical response and remission rates in ulcerative colitis patients. Thus, we believe that the detailed investigation of various studies on clinical trials may provide important information for the selection of appropriate biological agents, and therefore, we have extensively reviewed such trials in the present study.

Key words: Ulcerative colitis; Immune dysfunction; Biological therapy; Remission; Clinical trial; Inflammatory bowel disease

Core tip: In the last two years, the use of the Janus kinase 3 inhibitor (oral tofacitinib) and the α4β7 integrin blocker (vedolizumab) reportedly achieved good clinical response and remission rates in ulcerative colitis patients. Thus, we believe that the detailed investigation of various studies on clinical trials performed thus far may provide important information for the selection of appropriate biological agents, and therefore, we have extensively reviewed such trials in the present study.

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INTRODUCTION

Inflammatory bowel disease (IBD), which is broadly classified as ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic intestinal inflammation. Although its causes have not been clearly understood, it is believed to be influenced by genetic susceptibility, changes in the commensal enteric flora, and immune imbalance[1-5]. Increase in the production of T cells, cytokines, and chemokines, as well as increased trafficking of immune cells is associated with immune imbalance, and is considered as therapeutic targets for the biological treatment of IBD[6-9]. The current treatment goals of UC include induction of clinical remission, maintenance of clinical remission, and prevention of UC-related complications[10,11].

Although the therapeutic agents of UC, such as aminosalicylates, corticosteroids, thiopurines, and cyclosporine, are effective in most cases, biological agents are needed in cases where the disease is refractory or intolerant to therapeutic agents. Anti-tumor necrosis factor (TNF)-α agents are biological agents that are relatively safe and have been utilized for a long duration in UC patients. Anti-TNF-α agents appear to exert their effects by inhibiting the large amount of TNF-α present in the

Table 1 Summary of ACT1, ACT2, ULTRA1 and ULTRA2 trials for moderate-to-severe ulcerative colitis

Trial	Clinical scenario	Drug	Dosage	Patients (*n*)	Follow-up (wk)	Outcome and *P* value
ACT1 Rutgeerts *et al*[12]	Moderate-to-severe active UC	Infliximab	5 mg/kg IV 10 mg/kg IV (intravenously at weeks 0, 2, and 6 and then every eight weeks)	121 122	54	Clinical response at week 8 (placebo/5 mg/10 mg) -37.2%/69.4%/61.5% ($P < 0.001$, $P < 0.001$) Clinical remission at week 8 -14.9%/38.8%/32.0% ($P < 0.001$, $P = 0.002$) Clinical remission at week 54 -8.9%/25.7%/16.4% ($P = 0.006$, $P = 0.149$)
ACT2 Rutgeerts *et al*[12]	Moderate-to-severe active UC	Infliximab	5 mg/kg IV 10 mg/kg IV	121 120	30	Clinical response at week 8 (placebo/5 mg/10 mg) -29.3%/64.5%/69.2% ($P < 0.001$, $P < 0.001$) Clinical remission at week 8 -5.7%/33.9%/27.5% ($P < 0.001$, $P < 0.001$) Clinical remission at week 30 -3.3%/18.3%/27.3% ($P = 0.010$, $P < 0.001$)
ULTRA1 Reinisch *et al*[15]	Moderate-to-severe active UC	Adalimumab	160/80 mg SC (160 mg at week 0, 80 mg at week 2, 40 mg at weeks 4 and 6) 80/40 mg SC (80 mg at week 0, 40 mg at weeks 2, 4 and 6)	130 130	8	Clinical remission at week 8 (placebo/ADA160/80mg/ADA80/40 mg) - 9.2%/18.5%/10.0% ($P = 0.031$, $P = 0.833$)
ULTRA2 Sandborn *et al*[16]	Moderate- to-severe active UC	Adalimumab	160/80 mg SC (160 mg at week 0, 80 mg at week 2, and then 40 mg every other week)	248	52	Clinical remission at week 8 (placebo/ADA) - 9.3%/16.5% ($P = 0.019$) Clinical remission at week 52 - 8.5%/17.3% ($P = 0.004$)

UC: Ulcerative colitis; ADA: Adalimumab.

deeper layers of colonic tissues in UC patients[7]. In addition to anti-TNF-α agents, many studies have been performed on the use of molecules involved in varied inflammatory pathways as biological agents. However, in the present study, we aimed to focus on the mechanism of action, clinical outcomes, and future prospects of infliximab, adalimumab, and golimumab (anti-TNF-α agents); tofacitinib (a Janus kinase (JAK) 3 inhibitor); and vedolizumab (an α4β7 integrin blocker).

ANTI-TNF-α AGENTS: CLASSIC OR NEW GENERATION

Infilximab and adalimumab, the most commonly used anti-TNF-α agents at present, are administered intravenously and subcutaneously, respectively, and have been found to be effective for the treatment of moderate-to-severe UC in clinical trials. Anti-TNF-α, which was first officially approved by the US Food and Drug Administration (FDA) for the treatment of CD in 1998, was also approved by the FDA for the treatment of UC in 2010.

Both infliximab - chimeric mouse-human recombinant monoclonal antibody (25% murine and 75% human) - and adalimumab - completely human anti-TNF-α IgG1 - exert their effects by binding to free and membrane-bound TNF-α in order to prevent TNF-α from attaching to TNF-receptor type1/receptor type2. In ACT1 and ACT2 studies that reported the effects of infliximab in UC patients[12], no difference in the effect was noted between the 5-mg/kg administration group and the 10-mg/kg administration group, although a marked improvement was observed in the clinical response and remission rates after 8 wk ($P < 0.001$, Table 1), along with a significant improvement in mucosal healing ($P < 0.001$). Similar effects in the clinical remission rate were observed after 30 and 54 wk of treatment (Table 1). No differences in adverse effects were noted between the infliximab and placebo groups. Moreover, infliximab is well known as a useful rescue therapy to avoid colectomy[13,14].

In the first 8-wk multicenter randomized controlled study that utilized adalimumab, defined as ULTRA 1, the subjects were divided into 160/80 mg and 80/40 mg groups, based on the loading dose, and were then compared with the placebo group[15]. The clinical remission rate at week 8 in the adalimumab 160/80 mg group was 2 times higher than that of the placebo group, whereas this value in the adalimumab 80/40 mg group did not differ from that of the placebo group. Thereafter, a 52-wk randomized controlled study, defined as ULTRA2, was performed, which indicated that the clinical remission rate at week 52 in the adalimumab 160/80 mg group was 2 times higher than that of the placebo group ($P = 0.004$, Table 1)[16]. Following a subanalysis, it was observed that the anti-TNF-α naïve patient group exhibited approximately 2 times higher clinical remission rates at week 8 and week 52, respectively compared to the placebo group (21.3% *vs* 11.0%, 22.0% *vs* 12.4%) In the recently performed study on the effects of adali-

Table 2 Studies for the use of Golimumab, Tofacitinib and Vedolizumab for the treatment of ulcerative colitis

Trial	Clinical scenario	Drug	Dosage	Patients (*n*)	Follow-up (wk)	Outcome and *P* value
PURSUIT-SC Sandborn *et al*[23]	Moderate-to-severe active UC	Golimumab	100/50 mg SC 200/100 mg SC 400/200 mg SC (2 wk apart)	71 331 331	6	Clinical response at week 6 (placebo/GLM 200/100 mg/GLM 400/200mg) - 30.3%/51.0%/54.9% ($P < 0.0001$, $P < 0.0001$) Clinical remission at week 6 - 6.4%/17.8%/17.9% ($P < 0.0001$, $P < 0.0001$)
PURSUIT-Maintenance Sandborn *et al*[24]	Moderate-to-severe active UC	Golimumab	50 mg SC 100 mg SC (every 4 wk)	151 151	54	Clinical response at week 54 (placebo/GLM 50 mg/GLM100 mg) - 31.2%/47.0%/49.7% ($P = 0.010$, $P < 0.001$) Clinical remission at weeks 30 and 54 - 15.6%/23.2%/27.8% ($P = 0.122$, $P = 0.004$)
Sandborn *et al*[32]	Moderate-to-severe active UC	Tofacitinib	0.5 mg, 3 mg, 10 mg, 15 mg oral (twice daily for 8 wk)	31/33/33/49	8	Clinical response at week 8 (placebo/0.5 mg/3 mg/10 mg/15 mg) - 42%/32%/48%/61%/78% ($P = 0.39$, $P = 0.55$, $P = 0.10$, $P < 0.001$) Clinical remission at week 8 -10%/13%/33%/48%/41% ($P = 0.76$, $P = 0.001$, $P < 0.001$, $P < 0.001$) Endoscopic remission at week 8 -2%/10%/18%/30%/27% ($P = 0.14$, $P = 0.001$, $P < 0.001$, $P < 0.001$)
Gemini 1 Feagan *et al*[41]	Moderate-to-severe active UC	Vedolizumab	300 mg IV (at weeks 0, 2 and then every 8 or 4 wk)	Cohort 1 (225) Cohort 2 (521)	52	Clinical response at week 6 (placebo/Vedolizumab) - 25.5%/47.1% ($P < 0.001$) Clinical remission at week 6 - 5.4%/16.9% ($P = 0.001$) Clinical remission at week 52 (placebo/Vedolizumab every 8 wk/Vedolizumab every 4 wk) - 15.9%/41.8%/44.8% ($P < 0.001$, $P < 0.001$)

UC: Ulcerative colitis; GLM: Golimumab.

mumab on hospitalization for UC, the first 8 wk of adalimumab therapy indicated a significant reduction in the risk of all-cause, UC-related, and UC- or drug-related hospitalization compared to the placebo group (40%, 50%, and 47%, $P < 0.05$ for all comparisons)[17]; however, significant differences were not observed in the rates of colectomy between the groups. The adalimumab and placebo groups did not show any differences in the adverse events[15,16,18]. The primary failure rate of anti-TNF induction therapy is reportedly 40% in IBD clinical trials; when switching to another anti-TNF agent, the treatment becomes effective at 50%[19]. A secondary loss of response can also occur at 1 year after anti-TNF initiation in IBD patients[19], and solutions for the issues in anti-TNF-α treatment of UC are expected to be elucidated in the future.

Golimumab - a novel, completely human IgG1 anti-TNF-α antagonist - is subcutaneously administered and is approved for use in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis patients[20-22]. As shown in the PURSUIT-SC study, at week 6, the clinical response and remission rates showed a noticeable change in both the golimumab 200/100 mg and 400/200 mg groups (all $P < 0.0001$, Table 2)[23]. The PURSUIT- maintenance study, which is a phase 3, placebo-controlled, randomized withdrawal study, compared the clinical response and remission rates between the golimumab 50/100 mg group and placebo group up to week 54 at intervals of 4 wk; they observed that a notable change was observed in the golimumab 100 mg administration group ($P < 0.001$, $P = 0.004$, Table 2)[24]. After adjusting for the follow-up duration, no difference was noted in adverse events between the placebo and golimumab 100 mg groups.

PROMISING JAK1/JAK3 INHIBITOR AND INTEGRIN BLOCKING ANTIBODY

The JAK-STAT pathway is associated with inflammation, autoimmune diseases, hematopoietic disorders, and transplant rejection[25-30]. Tofacitinib (formally known as CP-690550) is a selective oral inhibitor of JAK 1 and 3, which is known to inhibit the differentiation of pathogenic Th1 and Th17 cells and innate immune cell signaling[31]. The effects of tofacitinib in active UC patients showed clinical response rates at week 8 of 32%, 48%, 61%, and 78% for the 0.5 mg, 3 mg, 10 mg, and 15 mg twice daily groups, respectively. The tofacitinib 3 mg, 10 mg, and 15 mg twice daily groups exhibited marked differences in clinical and endoscopic remission rates compared to the placebo group (all $P \leqslant 0.001$, Table 2)[32]. The levels of low-density and high- density lipoprotein cholesterol increased in a dose-dependent manner, and an absolute neutrophil count of < 1500 was observed in 2% of patients in the tofacitinib group. Thus, tofacitinib is considered to be an effective and safe drug for moderate-to-severe UC patients.

α4β7 integrin, a molecule that is expressed on circulating B and T lymphocytes, interacts with the ligand of the mucosal addressin-cell adhesion molecule

(MAdCAM-1)[33,34]. The bound lymphocyte migrates to the lamina propria and tissues, and then induces the inflammatory cascade[35]. Vedolizumab - a humanized monoclonal antibody that inhibits the binding of α4β7 integrin complex and MAdCAM-1-selectively blocks gut lymphocyte trafficking[36,37], and thus demonstrates therapeutic effects in IBD patients[38,39]. Consequently, unlike natalizumab which is a α4β7 and α4β1 integrin antagonist, vedolizumab does not affect the cerebrospinal fluid T-lymphocyte immunophenotype and therefore, it does not cause progressive multifocal leukoencephalopathy[40]. When vedolizumab (300 mg) was administered at week 0 and week 2 and then administered at intervals of 4 or 8 wk, a marked response in the clinical response and remission rates was noted after week 6 ($P < 0.001$, $P = 0.001$, respectively, Table 2)[41]. A noticeable change in the clinical remission rate at week 52 was observed, regardless of whether the medication was administered at 4- or 8-wk intervals (all $P < 0.001$).

CONCLUSION

Biological agents have been used for UC treatment for 10 years, and various types of biological agents have been developed and used worldwide, with the most common being anti-TNF-α agents. This increase in the development of biological agents provides further immunologic information in addition to offering a wide range of drugs for use. Thus, biological agents may serve as another appropriate option for clinicians in the treatment of UC patients who may not be effectively treated with conventional drugs. Since an accurate understanding of biological agents can be achieved through clinical trials, performed as part of large-scale randomized controlled studies, we have reviewed them in detail. In the future, we believe that biological agents with superior therapeutic effects and fewer side effects, compared to those used currently, will be developed, thus bridging the therapeutic gap present in the treatment of UC patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Enteral stents for the management of malignant colorectal obstruction

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Abstract

Colorectal cancer (CRC) is the 3rd most common cancer in the United States with more than 10000 new cases diagnosed annually. Approximately 20% of patients with CRC will have distant metastasis at time of diagnosis, making them poor candidates for primary surgical resection. Similarly, 8%-25% of patients with CRC will present with bowel obstruction and will require palliative therapy. Emergent surgical decompression has a high mortality and morbidity, and often leads to a colostomy which impairs the patient's quality of life. In the last decade, there has been an increasing use of colonic stents for palliative therapy to relieve malignant colonic obstruction. Colonic stents have been shown to be effective and safe to treat obstruction from CRC, and are now the therapy of choice in this scenario. In the setting of an acute bowel obstruction in patients with potentially resectable colon cancer, stents may be used to delay surgery and thus allow for decompression, adequate bowel preparation, and optimization of the patient's condition for curative surgical intervention. An overall complication rate (major and minor) of up to 25% has been associated with the procedure. Long term failure of stents may result from stent migration and tumor ingrowth. In the majority of cases, repeat stenting or surgical intervention can successfully overcome these adverse effects.

Key words: Colorectal cancer; Colonic obstruction; Self expanding metal stents; Intestinal obstruction/etiology; Intestinal obstruction/mortality; Intestinal obstruction/surgery; Survival rate

Core tip: Colonic stents are of benefit both as a bridge to surgery and as definitive therapy for colorectal obstruction in a large group of patients. Careful patient selection is required. Patients should be carefully managed in conjunction with the oncologist and surgeon. Endoscopists should also be vigilant for acute and delayed complications associated with colonic stent deployment.

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INTRODUCTION

A common complication of colorectal neoplasms is malignant large bowel obstruction. In the past, this complication was primarily managed with surgical resection; however, over the past 20 years endoscopic stenting has become an alternative, non-surgical approach. The first

Table 1 Commercially available self-expandable metal stent for malignant colonic obstruction

Manufacturer model	Delivery system	Diameter (mm)	Flares/flanges	Length (mm)	Covered/uncovered
WallFlex[1] (Boston Scientific)	TTS	22/27	Present	60, 90, 120	Uncovered
Ultraflex Precision[1] (Boston Scientific)	OTW	25/30	Present	57, 87, 117	Uncovered
Wallstent endoprosthesis[1] (Boston Scientific)	TTS	20, 22; no flare	Absent	60, 90	Uncovered
D-Enteral Colonic Stent (Taewoong Medical)	TTS/OTW	18, 20, 22, 24, 26	Absent	60, 80, 100, 120, 140, 150	Uncovered
Comvi Colonic Stent (Taewoong Medical)	TTS/OTW	18, 20, 22, 24, 26, 28	Absent	60, 80, 100, 120	Partially Covered
S-Enteral Colonic Stent (Taewoong Medical)	TTS/OTW	18, 20, 22, 24, 26, 28	Present	60, 80, 100, 120, 140, 150, 230	Fully and Partially Covered
Evolution Colonic Stent (Cook Endoscopy)	TTS	25	Present	60, 80, 100	Uncovered
Colonic Z-Stent[1] (Cook Endoscopy)	TTS	25	Present	40, 60, 80, 100, 120	Uncovered
Hanarostent (M.I.Tech)	TTS/OTW	20, 22, 24	Present	60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180	Uncovered, Fully Covered
Enterella (ELLA-CS)	OTW	22, 25, 30	Absent	75, 82, 88, 90, 112, 113, 123, 135, 136	Uncovered, Covered
Bonastent (EndoChoice)	TTS	22, 24, 26	Absent	60, 80, 100	Uncovered, Partially Covered
Aixstent (Leufen Medical)	TTS/OTW	25, 30	Present	80, 100	Uncovered, Partially Covered
Micro-Tech (MICRO-TECH Europe)	TTS/OTW	20, 25, 30	Present	60, 80, 100, 120	Uncovered, Partially Covered, Fully Covered

[1]Colonic stents availabe in the United States. TTS: Through-the-scope; OTW: Over-the wire.

studies demonstrating successful stenting of the colon to relieve malignancy-related obstruction were done in the early 1990s[1,2]. Since that time, the use of self-expanding metal stents (SEMS) has been implemented more frequently both as a bridge to surgical resection, as well as for palliation in advanced colorectal cancer and in patients who are poor surgical candidates. This review will discuss the types of stents currently available on the market, techniques for placement, indications, and adverse effects of colonic stenting in colorectal cancer.

TYPES OF SEMS

Currently, there are over a dozen different types of colonic SEMS available commercially worldwide (Table 1). These stents can be covered, partially covered, or uncovered and range from 20-30 mm in diameter with lengths of 6-18 cm. In the United States, only uncovered stents are approved for use in the large bowel, primarily because they have been shown to have a lower rate of migration when compared to covered stents. Studies have shown a migration rate among patients who received uncovered stent was 0%-2% as compared to 20%-40% among those with covered stents[1-4]. Uncovered stents may have a lower risk of other complications, such as stent fracture, failure of expansion, and loss of stent function[4,5]. Additionally, using uncovered stents improves the success rate of post-stent colonoscopies, which can be performed to evaluate for synchronous tumors following the diagnosis of colorectal cancer[6]. Finally, uncovered stents may be technically easier to deploy into more distal areas of obstruction, because they tend to use a smaller delivery systems and are less rigid.

The one major disadvantage of uncovered stents is the higher frequency of tumor ingrowth, which can precipitate stent occlusion[1,2,4]. Recent research has focused on developing colonic stents that would maintain their position, but also have the ability to prevent significant tumor in-growth. Moon *et al*[7] looked at outcomes of using a novel double-layered combination covered stent, with an internal membrane to prevent tumor in-growth and an external uncovered wire that should embed itself into the surrounding tumor. However, this stent, still showed an increased rate of migration when compared to uncovered stent, although not as high as documented in prior studies. Therefore, further innovation is necessary to design a stent that is a combination of covered and uncovered components to optimize its efficacy.

INDICATIONS FOR SEMS PLACEMENT

The high frequency with which colorectal cancer presents with malignant obstruction has created a growing use for self-expanding metal stents as palliation for malignant colonic obstruction as well as a bridge to surgery. Palliative stents offer the advantage of sparing patients surgical intervention which frequently results in colostomy. In patients for whom emergent surgical resection is planned, SEMS have been utilized as an efficacious method of delaying surgery and therefore reducing operative risk. Endoscopic stenting allows bowel decompression and sparing the patient emergent surgical decompression

which carries a mortality rate as high as 30%[8]. The low rate of morbidity and mortality associated with endoscopic colonic decompression when compared to surgery is therefore an attractive option.

Following the initial determination of the location and nature of the obstruction, a colonoscopy should be performed. Visualization of the site of obstruction provides the opportunity for a tissue biopsy as well as assessment of the potential for stent placement. Other considerations include the length of the stricture as well as the presence of an extrinsic versus intrinsic mass. If the colonoscope is easily passed through the site of obstruction, there is an increased risk for stent migration[9]. Stent placement is contraindicated in patients with perforation, intra-abdominal abscess, intestinal ischemia, or uncorrectable coagulopathy[10]. Placement of stents within 3-4 cm of the anal sphincter is not contraindicated but may be associated with increased incontinence and pain after placement, although in some patients a low lying stent in the rectum is their only clinical alternative to colostomy[11].

Palliative stents in patients with unresectable colorectal obstruction

Multiple comorbidities and metastatic disease are frequent contra-indications for surgical intervention to relieve malignant small bowel obstruction. In such situations, colonic stenting has been shown to be a safe and effective alternative approach for palliation[12]. In a retrospective, five-center study, Manes *et al*[13] looked at 201 patients with malignant large bowel obstruction who underwent palliative stenting and documented a 91.5% technical success rate and an 89.7% clinical success rate, defined as colonic decompression after 72 h. Another prospective study found a technical success rate of 95% and clinical success rate of 81%, which was defined as continued stent patency 6 mo after initial placement[14].

Although outcomes with SEMS appear to be quite good, there are few studies that directly compare colonic stenting with surgical resection. The data that is available indicates that utilizing SEMS confers certain benefits over surgical intervention, although the data pertaining to long term morbidity and mortality remains equivocal. Law *et al*[15] found that patients who underwent palliative colonic stenting had fewer admissions to the ICU and a lower likelihood of ultimately requiring resection with stoma creation as compared to the group that underwent surgery initially. Several other studies also showed statistically significant reduction in length of hospitalization, fewer short term complications, and decreased frequency of the need for stoma formation[15-17]. However, none of these studies showed any difference in overall survival between the two groups.

Colonic stents as a bridge to surgery

In the setting of an acute bowel obstruction in patients with potentially resectable colon cancer, stents may be used to delay surgery and thus allow for decompression, adequate bowel preparation, and optimization of the patient's condition for curative surgical intervention. Systematic reviews of stent placement as bridge to surgery have shown a technical success rate of 85%-92%[18]. Watt *et al*[19] performed a meta-analysis that included 88 studies and 1785 patients with 1845 stents placed. A total of 782 (43%) had stent placement as a bridge to surgery. Technical success and clinical success were reported to be 96.2% and 92% respectively. Data also suggested that placing colonic stents as a bridge to surgery increased the likelihood that the resection and re-anastomosis could be done as a one-stage procedure without the need for stoma formation when compared to an emergent operation. These studies documented that the one-step surgery was successful in 65%-73% of patients[20,21].

Few studies have compared colonic stenting as a bridge to surgery with emergent surgical decompression. Ng *et al*[21] performed a case-matched study of 20 patients who underwent SEMS as a bridge to surgery compared to 40 patients who had emergent surgical decompression. Patients in the stent group had statistically higher rates of primary anastomosis and shorter hospital and ICU stays. No difference was seen in mortality rates between the two groups.

In patients undergoing stent placement as a bridge to surgery in which it is later determined that surgery will not take place, the colonic stent may stay in place as a palliative measure. The benefits afforded by delaying surgery must be weighed against potential complications of stent placement which include such risks as perforation, bleeding, and stent migration. A study by Pirlet *et al*[22] comparing the stenting to surgery was stopped prematurely due to high rates of perforations in the stent group. A further source of uncertainty is whether stenting confers a survival benefit and decreased rate of stoma placement at 30 d. Further randomized control studies will be needed to determine the comparative clinical success of emergent surgery versus stenting as a bridge to surgery.

Techniques for stent placement

Colonic stent placement is a safe technique for the relief of large bowel obstruction secondary to malignancy. Recent studies have shown technical success rates that were close to 100% and clinical success rates, determined by the effective and persistent relief of the obstruction, of 85%-91%[23-25]. However, the complexity of the procedure can be significantly increased if the colon cannot be prepped before the intervention, if the obstruction is complete, and if the culprit lesion is located within a flexure or area of angulation. Additionally, the complexity of colonic stenting frequently requires the use of ERCP equipment and studies have shown that advanced endoscopists with pancreatico-biliary experience tend to have fewer complications and better outcomes[26]. Colonic stent placement can be performed by endoscopic guidance either using through-the-scope (TTS) or over-the wire (OTW) delivery systems. The former is ideal when using smaller diameter stents and for proximal lesions that cannot be accessed without an endoscope. The latter allows the delivery of larger stents and is most applicable

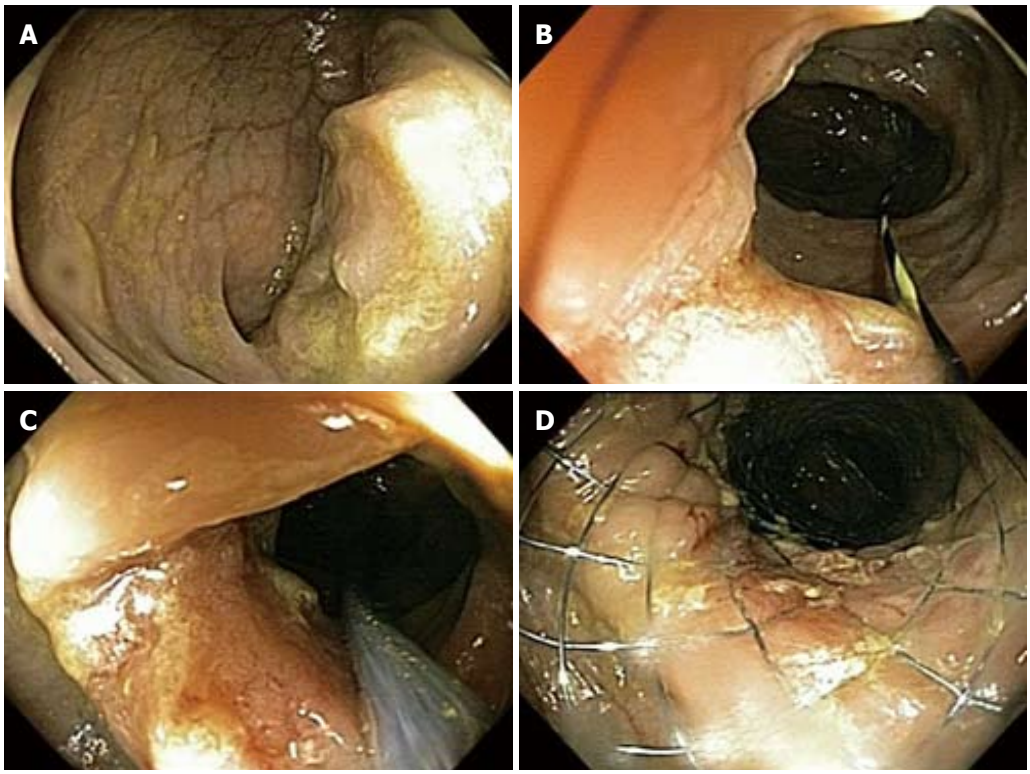

Figure 1 Through-the-scope approach has been shown to be safe and effective both in right-sided and left-sided colonic lesions. A: The patient presented with an obstructing colon cancer who was evaluated for an endoscopic colonic stent; B: The endoscope was passed to the site of the obstructive tumor and a 0.035 inch teflonated guide wire was passed across the stricture under endoscopic and fluoroscopic guidance; C: A catheter was subsequently passed over the guidewire; D: A colonic self-expanding metal stent (SEMS) was used to decompress the colon. The stent was placed so as to extend at least 2 cm on each end beyond the tumor margin. After the stent was deployed, the position was assessed by endoscopic and fluoroscopic visualization.

in cases of rectosigmoid obstruction.

The TTS approach has been shown to be safe and effective both in right-sided and left-sided colonic lesions (Figure 1)[27,28]. Almost all colonic stent deployed today use the TTS approach. Initially, an attempt should be made to pass the endoscope through the obstructing lesion, as this can facilitate the positioning of the guidewire and determination of the stricture length. The SEMS delivery system can then be passed over the guidewire and the stent deployed under direct endoscopic visualization. If the endoscope cannot be advanced beyond the obstruction, a hydrophilic biliary guidewire can be advanced through a biliary catheter to cannulate the lesion. Once fluoroscopy confirms that the guidewire has passed through the entirety of the obstruction and emerged on the proximal side, the stent is deployed[28]. The biliary catheters can also be used to aid in measurement of stricture length. One approach is to inflate the catheter balloon and position it at the distal end of the stricture. Subsequently, contrast is injected through the catheter and fluoroscopy is used to visualize the length of the stricture. Alternatively, the biliary catheter can be advanced all the way through the lesion and the balloon inflated. The catheter is then pulled back until resistance is met, indicating that it is now abutting the proximal end of the lesion. The endoscopist marked this point on the catheter by grasping it at the biopsy port exit point with his fingers. Then, the balloon is deflated and the catheter continues to be withdrawn until the balloon is visualized emerging at the distal end of the lesion. The distance between this point and the point marked by the endoscopist's fingers represents the length of the stricture[28]. Once the length of the stricture is determined, an appropriate SEMS needs to be selected. The ideal stent needs to extend at least 1-2 cm beyond the edge of the lesion on both proximal and distal ends to ensure that it will properly deploy and stay in place. When positioning the stent, the endoscopist needs to take into account shortening that occurs during deployment, as well as the rebound effect that occurs when the sheath is removed from the stent. After deployment, proper positioning is confirmed radiographically by an "hourglass" shape of the stent, with the ends beyond the obstruction fully expanded and the middle cinched by pressure from the lesion. Contrast can also be injected through the stent to confirm patency.

If the target lesion is in a difficult to reach location, such as a flexure or within an angulation of the colon, the endoscope may not be able to be positioned in a way that would allow successful passage of the guidewire. In such a situation a biliary sphincterotome can aid in the cannulation of the obstructing lesion[29-31]. The guidewire is loaded through the sphincterotome, which can then be rotated and bent in different directions until it positions the guidewire at an angle that would allow it to effectively traverse the extent of the obstructing lesion.

If the colonic obstruction is located in the descend-

ing colon, the non through-the-scope (OTW) approach can be used. This approach can be executed either under endoscopic guidance or with the aid of fluoroscopy. If the former method is used, rather than passing the SEMS through the working channel of the endoscope, the stent delivery system is advanced over a stiff guidewire, while the endoscope is passed next to it. This allows for direct visualization in order to make sure that the stent is properly deployed in the correct location. When this technique is employed, a colonoscope, which has a smaller diameter and is more flexible as compared to an upper endoscope, should be used. If the endoscopist chooses to use fluoroscopy, he or she first needs to mark the distal and proximal ends of the strictures with something that would be radiographically visible. Once this is done, the SEMS delivery system can be advanced over the guidewire to the site predetermined by the markers and deployed under fluoroscopic guidance.

Complications of SEMS

While self-expanding metal stents have been successfully used as a bridge to surgery in malignant obstruction of the colon as well as palliation, an overall complication rate (major and minor) of up to 25% has been associated with the procedure[18].

Vemulapalli *et al*[17] found more late-term complications in the stent group as compared to the surgical group, although this difference did not reach statistical significance. This finding was supported by data from several other studies that found that the late term complications in patients who received palliative SEMS was 24%-51%[17,26,32,33]. Factors that increased the risk of complications included operator experience, stent type, type of stricture, and tumor location as well as patient-related factors which include concommitant use of chemotherapy (specifically Avastin) and radiation. Complications may be grouped into minor and major categories

Minor complications of self-expanding metal stent placement

Bleeding, tenesmus, and pain are commonly reported following stent placement. Pain categorized as severe is seen in 5% of patients following stent placement. Pain and alterations in bowel habits is most often seen when stents are placed within 5 cm of the anal verge. Similarly, late-onset of tenesmus, pain, incontinence, and foreign body sensation may be seen with stent migration into the anorectal area[11,34]. However, Song *et al*[11] demonstrated that these symptoms typically resolve within 1 week or were responsive to analgesia.

Clinically mild bleeding is the most commonly-seen complication following SEMS placement with a reported incidence of 8%-12%. Bleeding can be attributed to friable mucosa associated with intrinsic masses as well as ulcerations and erosion and almost always resolves with conservative management[29]. Later bleeding can usually be attributed to ulcerations/erosions in the colonic mucosa.

Major complications

Stent migration: The incidence of covered stent migration significantly exceeds that of uncovered stents (50% *vs* 36%). Park *et al*[4] demonstrated that the lower rate of uncovered stent migration can be attributed to an increased rate of tumor ingrowth/infiltration. While frequently an adverse incidence, stent migration may herald improvement in a colonic stricture secondary to a response to therapy. Endoscopic removal of a covered stent may be performed in cases in which stent migration to the anorectal area results in pain or irritation. In the case of uncovered stent migration, argon plasma coagulation combined with rat-tooth forceps has been used[35]. Avoidance of stent migration is frequently achieved by placing the stent in the center of the stricture (or slightly above) and ensuring that it projects 2 to 4 cm proximally and distally into normal colon[18].

Perforation: The most serious complication of colonic SEMS placement is perforation, which has a reported mortality rate of 0.8% per stented patient[30]. Higher rates of complications have been reported in certain types of patients including those undergoing chemotherapy[36-38]. Faragher *et al*[16] reported an increased complication rate of 34.8% in patients receiving bevacizumab following stenting as compared to a 22.8% in untreated patients. This association may have also contributed to the high perforation rate in the stented group that lead to the premature termination of the only randomized controlled trial that attempted to compare outcomes between surgical intervention and SEMS in stage IV colon cancer patients on chemotherapy[35]. Chemotherapeutic agents, in particular bevacizumab, have been linked to an increased risk of colonic perforation[35].

Tumor overgrowth and ingrowth

Delayed stent obstruction due to tumor ingrowth or overgrowth is the most common complication following stent placement, with a reported incidence of up to 10%[10,37]. As the described above, the rate of tumor ingrowth is reduced with the use of covered stents. However, these SEMS have not been approved for use in the United States due to a higher risk of stent migration. Management options for stent re-obstruction include ablation, argon plasma coagulation, surgery and stenting. Repeat stenting with a stent-within-stent approach is the most common treatment approach and is almost always successful[39-42]. Far less common is stent failure secondary to stent collapse or stent fracture. van Hooft *et al*[38] demonstrated an increased incidence of stent fracture when used to treat benign obstructions.

Operator factors: As is seen with a multitude of different medical procedures, operator experience is inversely related to rates of complications in the placement of SEMS[34]. Colonic stenting is frequently performed on patients with extensive co-morbidities in acute settings. Consequently, the insufflation of air and manipulation of the different apparatuses will often result in higher rates of colonic perforation, the most serious complication associated with stent placement[9,10,37,43]. Some authors have

advocated use of carbon dioxide during colonic stenting as a preventive measure to reduce the risk of perforation.

Patient-related factors

Higher rates of complication including treatment failure have been observed in patients with extrinsic lesions[36]. Similarly, patients with longer segments of obstruction requiring stents longer than 10 cm had shorter event-free survival[44]. This can be attributed to the increased difficulty of placing these SEMS. Nonetheless, dilation of the bowel prior to stent deployment is not advisable given the risk of tumor fracture. Data regarding complication rates related to tumor location have been conflicting with no strong indication that SEMS placement in the right colon is unsafe.

CONCLUSION

Self-expanding metal stents are increasingly being used as palliation and as bridge to surgery in patients with malignant obstruction of the colon. With high technical and clinical success rates, endoscopic stenting provides a viable alternative to surgery. When used for these purposes colonic stents have the potential to decrease morbidity and cost as well as increase patient quality of life. Further randomized control studies will be needed to compare the efficacy of SEMS placement to traditional surgical approaches in the treatment of malignant obstructions.

Numerous studies have demonstrated a low incidence of complications associated with SEMS placement. The majority of reported adverse events are minor and self-limiting including pain and bleeding. Colonic perforation remains the most serious complication. Long term failure of stents may result from stent migration and tumor ingrowth. In the majority of cases, repeat stenting or surgical intervention can successfully overcome these adverse effects.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Impact of proteolytic enzymes in colorectal cancer development and progression

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Abstract

Tumor invasion and metastasis is a highly complicated, multi-step phenomenon. In the complex event of tumor progression, tumor cells interact with basement membrane and extracellular matrix components. Proteolytic enzymes (proteinases) are involved in the degradation of extracellular matrix, but also in cancer invasion and metastasis. The four categories of proteinases (cysteine-, serine-, aspartic-, and metalloproteinases) are named and classified according to the essential catalytic component in their active site. We and others have shown that proteolytic enzymes play a major role not only in colorectal cancer (CRC) invasion and metastasis, but also in malignant transformation of precancerous lesions into cancer. Tissue and serum-plasma antigen concentrations of proteinases might be of great value in identifying patients with poor prognosis in CRC. Our results, in concordance with others indicate the potential tumor marker impact of proteinases for the early diagnosis of CRC. In addition, proteinases may also serve as potential target molecules for therapeutic agents.

Key words: Proteinase; Cathepsin; Plasminogen activator; Matrix metalloproteinase; Colorectal cancer; Adenoma; Invasion; Metastasis; Biomarker; Prognosis

Core tip: Tumor invasion and metastasis is a highly complex phenomenon. Proteolytic enzymes (proteinases) are involved in the degradation of extracellular matrix, in colorectal cancer (CRC) invasion and metastasis, as well as in the malignant transformation of colorectal adenomas. Tissue and serum-plasma antigen concentrations of proteinases are strong prognostic factors in CRC and may have tumor marker impact for early diagnosis. Proteolytic enzymes may serve as potential target molecules for CRC therapy. Their use in combination with established chemotherapeutic strategies might have the potential to become a valuable oncological treatment modality.

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MECHANISMS OF TUMOR PROGRESSION

Tumor invasion and metastasis is a highly complicated, multi-step phenomenon. The multistep metastatic process requires the actions of several genes and involves the

Table 1 Categories and main properties of proteolytic enzymes

Property	Cysteine proteases	Serine proteases	Aspartic proteases	Metallo proteases
Active site	Cysteine	Serine	Aspartic acid	Zinc
Optimum pH	7-9	3-7	2-4	5-9
Location	Lysosomes	Intra-extracellular	Lysosomes	Intra-extracellular
Examples	Cathepsins	Elastase	Pepsinogen I - II	Collagenase
	B, L, C, H	Trypsin	Cathepsins D, E	Stromelysin
		PA: uPA, tPA	Gelatinase	Matrilysin
Inhibitors	Cystatin	Antitrypsin Antithrombin III PAI	Unknown	TIMP

PA: Plasminogen activators; uPA: Uroinase-type plasminogen activator; tPA: Tissue-type plasminogen activator; PAI: Plasminogen activator inhibitor; TIMP: Tissue inhibitor of metalloproteinases.

initial transforming event (*i.e.*, oncogene activation), proliferation of transformed cells and the ability of tumor cells to avoid destruction by immune-mechanisms. Furthermore, it comprises of nutrition supply to the tumor mass by the release of tumor angiogenesis factors, cancer cell local invasion and destruction of extracellular matrix (ECM) components, epithelial mesenchymal transition (EMT), shedding from primary tumor, intravasation, arrest, extravasation and colonization at a preferential site resulting in the formation of a secondary tumor (distant metastases)[1-9].

Chronic and persistent inflammation can predispose to carcinogenesis and contribute to cancer development. Cancer-associated inflammation includes infiltrating leucocytes, cytokines, chemokines, growth factors and matrix-degrading enzymes. Inflammatory conditions can initiate oncogenic transformation and epigenetic and genetic changes in malignant cells. In essence, two interrelated pathways connect inflammation and cancer: (1) genetic alterations (including chromosomal amplification, activation of oncogenes and inactivation of tumor-suppressor genes) leading to neoplastic transformation; and (2) presence of tumor-infiltrating leukocytes that are prime regulators of cancer-related inflammation. The integration of these two pathways activates transcription factors and finally creates a tumor-associated inflammatory milieu[10-19].

BASEMENT MEMBRANE, EXTRACELLULAR MATRIX AND CANCER CELLS

All normal or pre-invasive tumor epithelia are physically segregated from vascular structures within the stroma by the basement membrane (BM). The BM consists of laminins, type Ⅳ collagen and surrounding epithelial cells. The tumor stroma is comprised of ECM, non-malignant cells, and the signalling molecules they produce. During tumor invasion and metastasis, tumor cells are interacting with the BM and the ECM. The disruption of the BM and the ECM is an essential pre-requisite for cancer cell invasion and metastasis. Interaction of tumor cells with the BM and ECM comprises of three steps: attachment, matrix dissolution and migration. The first step is tumor cell attachment to the matrix. The attachment is mediated by tumor cell surface receptors, when tumor cells bind to BM surface. This process involves specific glycoproteins such as fibronectin, type Ⅳ collagen and laminin. In the second step tumor cells directly secrete degradative enzymes or induce the host to produce proteolytic enzymes to degrade ECM. The matrix lysis takes place in a highly localized region close to the tumor cell surface. During the third step (migration), cancer cells are propelled across the BM and stroma through the zone of matrix proteolysis. Invasion of ECM is accomplished by reverberation of these three steps[3,6,9,20-27].

Table 2 General roles of proteolytic enzymes in cancers

Degradation or disruption of basement membrane and extracellular matrix
Produce components which allow the *in situ* cancer cells to disseminate to distant organ
Formation of a complex microenvironment that promotes malignant transformation
Activation of growth factors, adhesion molecules
Suppression of tumor cell apoptosis
Destruction of chemokine gradients
Modulation of antitumor immune reactions
Dual and complex role in angiogenesis

GENERAL ROLES OF PROTEOLYTIC ENZYMES IN CANCERS

The four categories of proteinases (cysteine-, serine-, aspartic-, and metalloproteinases) are named and classified according to the essential catalytic component (usually an amino acid) in their active site[3,28]. Table 1 summarises the four categories and some general properties of each group. Proteolytic enzymes play a major role in the breakdown and reconstitution of ECM in a variety of physiological and pathological processes, such as protein turnover, tissue remodeling, wound repair, angiogenesis, destructive diseases, inflammatory disorders as well as tumor invasion and metastasis (Table 2). Tumor cells have been shown to produce and release several proteolytic enzymes, which are thought to be involved in tumor invasion and metastasis. Proteolytic enzymes are also frequently produced by surrounding stromal cells, including fibroblasts and inflammatory cells. It has been proposed that these proteolytic enzymes cause degradation or disruption of BM and ECM components allowing the *in situ* cancer cells to migrate into the adjacent stroma or to disseminate to distant organ. It is commonly accepted that progression from *in situ* to invasive or metastatic cancer is caused by proteolytic enzymes (proteinases) produced

by tumor cells that increase linearly in concentration with tumor progression[3,6,9,29-31].

The impact of proteolytic enzymes in tumor progression is much more complex than that derived from their direct degradative action on BM and ECM components. Now they are known to have functions that extend far outside matrix remodeling. Proteolytic processing of bioactive molecules by proteinases contributes to the formation of a complex microenvironment that promotes malignant transformation. Proteolytic enzymes can contribute to tumor growth either directly or indirectly via growth factors such as transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF) or, insulin-like growth factor (IGF). Proteinases also act on other non-matrix substrates (*e.g.*, chemokines, adhesion molecules, apoptotic mediators, angiogenic factors) that yield the critical cellular responses that are essential for tumor growth and progression. Proteolytic enzymes are also associated with a variety of escape mechanisms that tumor cells develop to avoid immune response including chemokine cleavage and regulation of chemokine mobilization. Tumor cells produce chemokines, cytokines, and the extracellular matrix metalloprotease inducer (EMMPRIN), which in turn activates tumor-cell invasion. During angiogenesis, proteolytic enzymes can have both a pro-angiogenic impact, by releasing matrix-bound pro-angiogenic factors such as TGF-β, bFGF, triggering the angiogenic switch during carcinogenesis and facilitating vascular remodeling and neovascularization at distant sites during metastasis, and also may have anti-angiogenic role, by cleaving the ECM components into anti-angiogenic factors[32-36].

Cysteine and serine proteinases

It has been observed that cysteine proteinases [cathepsin B (CATB) and cathepsin L (CATL)], play a crucial role in the destruction of various elements of the cell-surrounding ECM. The serine proteinase urokinase-type plasminogen inhibitor (uPA) is also involved in many protein degrading processes. uPA seems to promote invasion through a plasmin mediated degradation of ECM proteins. Active uPA catalyzes the conversion from plasminogen to plasmin, which is a potent activator of several metalloproteinase proenzymes, such as prostromelysin, procollagenase, and progelatinase B. Beyond its direct proteolytic capacity, CATB has also been shown to activate the prourokinase-type plasminogen activator (pro-uPA). The tissue-type plasminogen activator (TPA) is a key enzyme in the fibrinolytic cascade. Plasminogen activators (PA) are controlled by plasminogen activator inhibitors, which are members of the serine proteinase inhibitors (serpin) family. The PA inhibitor type-1 (PAI-1) under normal physiologic conditions inhibits both uPA and TPA. The exact role of PAI-1 in tumor biology is complex: PAI-1 may represent a specific protein of transformed malignant tissue; may protect cancer tissue against the proteolytic degradation triggered by the tumor on surrounding normal tissue; and finally, PAI-1 has a role in angiogenesis playing a part in tumor spread[37-45].

Cathepsins and components of the plasminogen activator and inhibitor system have been demonstrated in various malignant tissues, *e.g.*, breast cancer[46-48], lung cancer[49,50], head and neck cancer[51], ovarian cancer[52] or gastric cancer[53-56] and might therefore be useful as a diagnostic tool.

With respect to the gastrointestinal (GI) tract, we have previously shown that cysteine and serine proteinases are widely distributed in GI tissues, being implicated in processes of GI tissue remodelling, angiogenesis, wound healing, inflammation, may have a role not only in the process of esophageal or gastric cancer invasion, but also in the progression of GI precancerous lesions into cancer[57-61]. Several studies, along with our own, have also pointed to the prognostic value of cysteine and serine proteinases for survival, for instance, in gastric cancer[56,58,59,62,63], pancreatic cancer[64], or hepatocellular carcinoma[65].

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) family consists of 26 members of homologous zinc-dependent endopeptidases. The expression of MMPs is induced by a variety of external stimuli such as cytokines and growth factors, including interleukins, interferons, vascular endothelial growth factor, fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α) or TNF-β, and EMMPRIN. MMPs play an important role in the degradation of ECM components, are crucial for tumor growth, invasion and metastasis. MMPs are synthesized as inactive zymogens, which are then activated by either other MMPs or by serine proteinases. Based on substrate specificities and sequence homology, MMPs can be classified as gelatinases, collagenases, stromelysins and matrilysins. MMPs are responsible for cleaving all of the major ECM proteins[66-74].

We and others have shown that MMPs, particularly type Ⅳ collagenase MMP-9 (gelatinase B), are essential in the process of tumor invasion and metastasis, as well as in the remodeling and inflammatory processes in IBD[75-82].

MMPs have also been considered as potential diagnostic and prognostic biomarkers in many types and stages of GI cancer[83-85].

Tissue inhibitors of matrix metalloproteinases

MMPs activity is specifically inhibited by natural inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Currently, four different TIMPs have been characterized in humans (TIMP-1, -2, -3 and -4). The balanced interaction of MMPs with TIMPs regulates ECM homeostasis. The imbalance between MMPs and TIMPs is an essential step in the development of malignancies. TIMPs might display a dual influence on tumor progression: either beneficial by inhibiting MMPs and impairing angiogenesis or harmful by facilitating cancer cell generation, tumor growth and metastasis. The sensitive balance between MMPs and TIMPs is essential for many physi-

Table 3 Clinical significance of proteolytic enzymes in colorectal cancer

Role in colorectal tumor biology, in the process of tumor invasion and metastasis
Role in malignant transformation of colorectal precancerous conditions and lesions
Potential diagnostic tool
New and independent prognostic factors
Potential tumor marker impact for early diagnosis
Potential target molecules for therapeutic agents

ological processes in the gut[6,86-91].

We have recently demonstrated that not only MMPs but also TIMPs may contribute to the inflammatory and remodeling processes in IBD and serum TIMP-1 might be useful as additional biomarker in the assessment of IBD activity[82].

IMPACT OF PROTEOLYTIC ENZYMES IN COLORECTAL CANCER

colorectal cancer (CRC) is the third most common malignant neoplasm worldwide. CRC is the second most common newly diagnosed cancer and the second most common cause of cancer death in the European Union (EU). Despite the advances in screening, diagnosis, and treatment, the overall long-term outcome of curatively resected patients has not significantly changed in the last decades, the five-year survival rate being approximately 60%. More than a half of CRCs are still diagnosed only when the disease involves regional or distant organs, and these patients are candidates for systemic chemotherapy. The prognosis of CRC is determined primarily by TNM staging and pathomorphological parameters. Furthermore, therapeutic strategies for chemotherapy are based on traditional prognostic systems. For risk stratification, it would be useful for clinicians to have new and more efficient preoperative tumor markers and prognostic indicators available, for instance to better identify patients who need adjuvant or neoadjuvant treatment[92-97].

We review hereinafter the prognostic value and potential tumor marker impact of a number of proteolytic enzymes. We also discuss proteinases as potential target molecules for therapeutic agents Table 3 summarises the clinical significance of proteolytic enzymes in CRC.

Tissue expression of cysteine and serine proteinases in CRC

Cathepsins or components of the plasminogen activator and inhibitor system have been demonstrated in colorectal cancer tissue and might therefore be useful as diagnostic tools[98-100]. Some of the studies also showed that cathepsins or some of PAs also have a prognostic significance for patients with CRC[98-106]. In a former study we have demonstrated the simultaneous up-regulation of both cysteine and serine proteinases in CRC confirming their role in colorectal tumor biology and particularly in the process of tumor invasion and metastasis. We have also shown that CATL, uPA and PAI-1 have a major prognostic impact in patients with CRC[107].

Serum and plasma concentrations of cysteine and serine proteinases in CRC

Given the lack in the literature of a comparison of the behavior of cysteine proteinase CATB and serine proteinase uPA in the same experimental setting in different GI tumors, in a previous study we have surveyed the possible clinical impact of serum CATB and plasma UPA antigen in CRC, gastric cancer, hepatocellular carcinoma, pancreatic cancer, and colorectal adenomas[61]. We have demonstrated that preoperative serum CATB and plasma uPA antigen concentrations were significantly higher in GI tract cancers overall than those found in control non-cancer patients, thus confirming the relevance of cysteine and serine protease-dependent mechanisms in GI cancers. We have also further confirmed that serum CATB and uPA plasma levels were elevated in patients with CRC[108-111]. In addition, we have shown that serum CATB and plasma uPA did show a significant increase in advanced CRC stage. We have also demonstrated for the first time that antigen levels of CATB and uPA were significantly higher in blood samples of patients with colorectal adenomas as compared to controls. Furthermore, we have found significantly higher CATB and uPA antigen levels in patients with tubulovillous adenomas with high-grade dysplasia (HGD) compared to those with tubular adenomas with low-grade dysplasia (LGD). Thus taken together, the data from blood samples with previous results obtained in colorectal tissues confirm that a concomitant activation of serine (CATB) and uPA may be involved in the progression from premalignant colorectal adenomas into CRC[112,113].

Some other studies have also suggested the potential impact of cysteine or serine proteases as tumor markers in CRC[108-111]. Given the lack in the literature for a comparison of the tumor marker utility and possible prognostic relevance of cathepsins (CATB, CATL) and the uPA/PAI-1 system in the same experimental setting, we have surveyed the behavior of CATB, CATL, uPA, PAI-1 in CRC and compared it with the commonly used gastrointestinal tumor markers CEA and CA 19-9, and then evaluated any correlation between these parameters and the clinico-pathological staging of CRC[114]. In this study, we have demonstrated the potential tumor marker utility and prognostic relevance of cysteine and serine proteinases for patients with CRC. We have also shown the concomitant activation of these systems in CRC. At the time of clinical presentation, proteinases were more sensitive indicators of diagnosis than the most commonly used markers CEA and CA 19-9. When proteinases, CEA and CA 19-9 were used as single markers, we found that their sensitivity was more indicative of CRC than CEA or CA 19-9. The simultaneous determination of several markers led to a higher sensitivity in our group of CRC patients: PAI-1 combined with CATB or

uPA was superior compared to the combinations of all other markers. In addition, the sensitivity of CEA or CA 19-9 in combination with a proteinase antigen level was more indicative for CRC than CEA or CA 19-9 alone. In this study increased serum or plasma proteinase concentrations significantly correlated with advanced tumor stage. In a univariate survival analysis high serum CATB, CATL and plasma PAI-1 antigen levels identified patients with shorter survival and those who were at higher risk of death. In addition, PAI-1 and CATB were proved as independent predictor variables in a multivariate statistical analysis. We also confirmed that cysteine and serine proteinases were significantly higher in blood samples of patients with colorectal adenomas compared to controls, suggesting that these proteinases may be involved in the malignant transformation of colorectal adenomas[114].

Tissue MMPs and TIMPs in CRC

The behavior of MMPs and TIMPs in CRC has recently been extensively reviewed by our own group[115]. Several studies have shown that the expression of several MMPs and TIMPs are enhanced in CRC. In an immunohistochemical study we have demonstrated that tissue expression of one particular MMP, MMP-9 was significantly higher in moderately (G2) and poorly (G3) differentiated tumors than in well differentiated (G1) cancers, as well as in advanced Dukes stages compared with Dukes stage A[116]. Some recent studies have confirmed that tissue MMP-9 can be considered as an independent prognostic marker in CRC[117-120]. In addition, it has been demonstrated that not only MMP-9, but also tissue expressions of other MMPs and TIMPs have strong prognostic impact in CRC[121-126].

MMPs and TIMPs also play a role in malignant transformation from colorectal adenomas to CRC. Our group in concordance with others has shown that tissue expression of MMP-9, MMP-2, TIMP-1 and TIMP-2 were significantly higher in advanced versus non-advanced adenomas, suggesting that MMPs and TIMPs might be markers for early colorectal carcinogenesis. The ability of MMPs and TIMPs to distinguish adenomas with HGD from adenomas without HGD may be of clinical value in predicting additional cancer risk for an individual patient[116,127-130].

Serum and plasma MMPs and TIMPs in CRC

The diagnostic, prognostic and tumor marker impact of serum-plasma MMPs and TIMPs in CRC has been extensively studied. We have demonstrated that serum antigen concentrations of MMP-2, MMP-7, MMP-9 as well as TIMP-1 and TIMP-2 were significantly higher in patients with CRC than in healthy controls. All examined parameters were significantly higher in patients with CRC than in patients with adenomas. Higher antigen concentrations of MMPs and TIMPs significantly correlated with preoperative tumor stage. The data from blood samples confirmed previous results of tissue expressions concluding that MMPs and their inhibitors TIMP-1 and TIMP-2 play an important role not only in CRC invasion and metastasis, but they are also activated in premalignant colorectal adenomas[82].

Several recent studies confirmed that high preoperative serum or plasma MMP-2, MMP-9 and mainly TIMP-1 antigen levels are strong predictive factors for poor prognosis in patients with CRC[131-137].

The potential tumor marker role of MMPs and TIMPs has also been extensively studied. It has been demonstrated that MMP-9 and TIMP-1 have significant potential as biomarkers in CRC. Diagnostic sensitivity of MMP-9 and TIMP-1 was consistently higher as compared with the conventional biomarkers (CEA or CA 19-9)[131,138-140].

It has also been proposed that TIMPs can predict individual response to chemotherapy and could be considered as an additional tool for monitoring chemotherapy in CRC[141-144].

One of the greatest challenges in CRC management is to predict the outcome of each patient so that we can determine who will really benefit from intensified adjuvant therapy. The classical TNM staging system relied heavily on the exact extent of cancer at the time of diagnosis and is greatly predictive in stage I and stage Ⅳ tumors. However, it is less informative for patients including stage Ⅱ and stage Ⅲ CRC. After curative surgery, stage Ⅲ CRC patients experience 50% chance of developing recurrence. It is well documented that the overall survival rate of stage Ⅲ CRC could clearly benefit from adjuvant chemotherapy. In contrast, the role of adjuvant chemotherapy for stage Ⅱ CRC is still controversial, despite the 20% recurrence in this group. We and others have suggested that proteolytic enzymes could constitute useful independent markers in addition to the TNM staging system for the intermediate groups including stage Ⅱ and stage Ⅲ CRC. Proteolytic enzymes may help to identify patients who are more likely to have disease relapse and high risk of death, thus those who are potential candidates to receive aggressive adjuvant chemotherapy[107,119,145-147].

On the other hand, taken into consideration that proteolytic enzymes may have a crucial role not only in the invasive process of CRC, but also in the progression of precancerous conditions and lesions into cancer, quantification of proteinases might be useful to identify patients at higher risk for progression to cancer, who could be subjected to a more strict follow-up protocol.

PROTEOLYTIC ENZYMES AS POTENTIAL TARGET FOR CANCER THERAPY

The accumulated evidence strongly supports the concept of the use of cysteine proteinases (cathepsins) and serine proteinases (uPA-PAI system) as targets for cancer therapy. It has been suggested that cathepsin inhibition represents a potential therapeutic strategy for the treatment of cancer. Cathepsins inhibition seems to reduce invasion and metastasis, but there is concern that selective cathep-

sin inhibition induces compensatory activity by other cathepsins. The combination of cathepsin inhibition with conventional chemotherapy seems to be more effective and has yielded more consistent clinical results. Future research should be focused on the exact mechanisms and clinical effects of this combination treatment[148-151].

The multifunctionality of the uPA-uPAR-PAI-1 system in cancer spotlighted serine proteinases to become potential targets for anticancer treatment. In addition, uPA system is also increasingly being recognized as a candidate target for gene therapy in cancer[152-159]. Several strategies, including the use of ribozymes, DNAzyme, antisense oligodeoxynucleotides, uPA inhibitors, soluble uPAR, catalytically inactive uPA fragments, the interactions of uPAR with integrins and transmembrane receptors, synthetic peptides and synthetic hybrids are under study, as they all interfere with the activity of uPA or uPAR in tumor cells. Many clinical studies are ongoing and some uPA-related compounds have reached Phase Ⅱ clinical trials.

Several therapeutic MMP inhibitors (MMPIs) have also been developed to target MMPs. Various natural compounds have been identified as inhibiting MMPs. In addition, several generations of synthetic MMPIs were tested in phase Ⅲ clinical trials in humans, including peptidomimetics, non-peptidomimetic inhibitors or tetracycline derivates, targeting MMPs in the extracellular space. Other strategies of MMP inhibition involve small interfering and antisense RNA technology[160-165]. In contrast to their promising effect in preclinical models, most of these agents unfortunately failed in clinical trials, thus they are yet not available for routine use. The use of broad-spectrum MMPIs may lead to undesired clinical consequences as a result of the wide range of MMPs that are inhibited. In addition, toxic side effects, such as musculoskeletal syndrome, have limited drug efficiency.

The ADAMs is also a family of potential new targets for cancer therapy. ADAMs (a disintegrin and metalloproteinase) are members of a zinc-dependent family of matrix metalloproteinases. Preclinical findings suggest that selective ADAM inhibitors might be novel anticancer agents. ADAMs inhibitors may be particularly useful in treating cancers that depend on HER or TNF-α-mediated signalling[166-169].

One of the major challenges for the future is the development of monoclonal antibodies or inhibitors that are specific for certain MMPs, showing no cross-reaction with other MMPs with improved pharmacokinetic properties and selectivity. In addition, their use in combination with established chemotherapeutic strategies might have the potential to become valuable oncological treatment modalities[161,170-176].

CONCLUSION

Proteolytic enzymes play a sophisticated role in cancer development and progression due to their abilities to degrade various substrates. However, the role of proteolytic enzymes in tumor progression is much more complex than that derived from their direct degradative action on BM and ECM components. Proteinases may have a crucial role not only in the invasive process of CRC, but also in the progression of precancerous conditions and lesions to CRC. Proteolytic enzymes could constitute effective independent prognostic markers additive to TNM staging system in CRC. Their determination might be useful to identify patients at higher risk for progression to cancer, who could be subjected to a more strict endoscopic follow-up protocol.

It has recently been demonstrated in experimental settings that a newly developed near-infrared bioactivable probe (MMPSense) that reports the activity of a broad array of MMP isoforms detects both polypoid and non-polypoid early colorectal adenomas with a high specificity[177]. Future studies will focus on the use of such fluorescent probes combined with colonoscopy to identify neoplastic lesions based on their molecular "fingerprint" (*i.e.*, proteinase enzymatic activity) rather than solely on their morphologic properties. The ability to detect non-polypoid lesions using this fluorescent probe alone or in combination with other molecular probes may offer new future perspectives for colorectal screening. In addition, it might also serve as a potential strategy for the pharmacodynamic monitoring of targeted therapy. The pharmacological targeting of CRC by the development of a new generation of effective and selective proteinase inhibitors is another emerging area of research.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Probiotics against neoplastic transformation of gastric mucosa: Effects on cell proliferation and polyamine metabolism

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Abstract

Gastric cancer is still the second leading cause of cancer death worldwide, accounting for about 10% of newly diagnosed neoplasms. In the last decades, an emerging role has been attributed to the relations between the intestinal microbiota and the onset of both gastrointestinal and non-gastrointestinal neoplasms. Thus, exogenous microbial administration of peculiar bacterial strains (probiotics) has been suggested as having a profound influence on multiple processes associated with a change in cancer risk. The internationally accepted definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The possible effects on the gastrointestinal tract following probiotic administration have been investigated *in vitro* and in animal models, as well as in healthy volunteers and in patients suffering from different human gastrointestinal diseases. Although several evidences are available on the use of probiotics against the carcinogen *Helicobacter pylori*, little is still known about the potential cross-interactions among probiotics, the composition and quality of intestinal flora and the neoplastic transformation of gastric mucosa. In this connection, a significant role in cell proliferation is played by polyamines (putrescine, spermidine, and spermine). These small amines are required in both pre-neoplastic and neoplastic tissue to sustain the cell growth and the evidences here provided suggest that probiotics may act as antineoplastic agents in the stomach by affecting also the polyamine content and functions. This review will summarize data on the most widely recognized effects of probiotics against neoplastic transformation of gastric mucosa and in particular on their ability in modulating cell proliferation, paying attention to the polyamine metabolism.

Key words: Cell proliferation; Gastric cancer; Microbiota; Polyamines; Probiotics

Core tip: Studies linking probiotics and gastric neoplasms have mainly been addressed to evaluate the potential of probiotics as alternative regimen against *Helicobacter pylori*, a carcinogen tightly connected to gastric cancer. The effects of probiotics on gastric cell proliferation are still under investigation and interest has also been paid on the polyamines metabolism. Polyamines (putrescine, spermidine and spermine) are pivotal in regulating different metabolic functions, including cell proliferation. In this review, the authors try to summarize data on gastric cancer and the proposed abilities by probiotics in affecting the gastric integrity and cell proliferation, also in relation to the polyamine metabolism.

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INTRODUCTION

In spite of the significant reduction in its incidence and mortality in the last four decades[1], gastric cancer (GC) remains the second leading cause of cancer death worldwide, accounting for about 10% of newly diagnosed neoplasms[2].

In the last years, an emerging role has been attributed to the possible connections between the gastrointestinal (GI) content and the onset of neoplasms[3]. In this framework, modifications in the composition and quality of intestinal flora have been considered as having a deep impact on the host activities, not only at a local level on the different intestinal metabolic traits, but also in a more systemic fashion (*e.g.*, by affecting the immune response or cell proliferation)[4]. Thus, administration of peculiar positive bacterial strains (probiotics) has been suggested as having a profound influence on multiple processes associated with a change in cancer risk[5].

Many different definitions for probiotics have been proposed over the years. A widely accepted definition for probiotics is "mono or mixed cultures of live microorganisms which when administered in adequate amounts confer a health benefit on the host"[6]. Among them, lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used[7]. To be metabolically active, these probiotic strains must survive passage through the GI tract, especially in the hostile gastric environment and resist to the enzymes of pancreas and bile acids. In this way they can reach the large intestine where they should be in sufficient amount to colonize mucosa and stools[8]. Besides, as stated by the international guidelines[9], for a reliable therapeutic use in humans, probiotics should be of human origin, safe for the host, and genetically stable.

The possible association between probiotics and GI neoplasms has mainly been evaluated in relation to colorectal cancer (CRC)[10].

In the stomach, studies linking probiotics and gastric neoplasms have been essentially addressed to evaluate the potential of probiotics as alternative regimen to eradicate *Helicobacter pylori* (*H. pylori*), a recognized carcinogen tightly connected to GC onset[11]. However, little is still known about the potential interactions between different strains of probiotics and the neoplastic transformation of gastric mucosa. In particular, the effects of probiotics on gastric cell proliferation are still under investigation and interest has also been paid on the polyamines metabolism. Putrescine, spermidine, and spermine are ubiquitous short-chain aliphatic amines involved in the regulation of cell proliferation and differentiation[12]. Polyamines can be considered reliable markers of proliferation since abnormal hyper-proliferative cells, such as in neoplastic and preneoplastic tissue, exhibit very high requirements for these amines to sustain cell growth through elevated DNA, RNA, and protein synthesis[13].

In this framework, this review will summarize data on gastric microbiota, the onset of GC and the proposed mechanisms of probiotics in contrasting the neoplastic transformation of the gastric mucosa. Additionally, attention will be focused on the possible influence of different probiotic strains on the polyamine metabolism.

HUMAN GASTRIC MICROBIOTA

The adult human GI tract contains 100 trillion microbial organisms, collectively denominated microbiota[14]. This microbe population is known to be dominated by strict anaerobes, including *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus*, and *Atopobium*. Anaerobic bacteria prevail aerobic bacteria by a factor of 100 to 1000 to 1 and include lactobacilli, enterococci, streptococci and *Enterobacteriaceae*. More than 500 different bacterial species have been described in the normal commensal microbiota, although the exact number and the variability among individuals are still object of deep investigations[15,16]. As reported in Figure 1, the bacterial density progressively increases along the proximal to distal segments of the GI tract and rises up to 10^{11} to 10^{12} bacteria per gram of colonic content[17].

The stomach is the least populated region of the GI tract (along with the esophagus and duodenum) and its microbial density ranges from 10 to 1000 CFU/g. Physiologically, this low number of bacteria in the gastric lumen has to be ascribed not only to low pH values, but also to rapid peristalsis and high bile concentrations. Different bacteria can regularly be sampled from the stomachs of healthy adults. Commonly detected phyla include Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria, and characteristic genera are *Lactobacillus*, *Streptococcus*, and *Propionibacterium*[18].

The discovery of the spiral-shaped Gram-negative *H. pylori* inverted the conventional dogma of the stomach as a sterile organ. Actually, the stomach is populated by different bacteria and *H. pylori* is able to profoundly affect gastric physiology and the properties of the gastric mucosa as an ecological niche for other microbes[19]. As a result, the composition of the microbial population in the stomach may vary according to the presence or not of *H. pylori*.

Andersson *et al*[20] investigated the mucosa-associated microbiota of six healthy stomachs in a culture-independent study based on short 16S rRNA gene sequence reads by 454 pyrosequencing technology. Three of the six study subjects were not infected by *H. pylori*. The composition of their stomach microbiota was different from individual to individual, and only 33 out of the 262 phylotypes found were contemporary detected in all three samples. The authors hypothesized that the most abundant of the phylotypes found were apparently swallowed organisms originating from the mouth or esophagus. However, they also identified 177 phylotypes that were found in *H. pylori*-free stomachs, but not in the throat

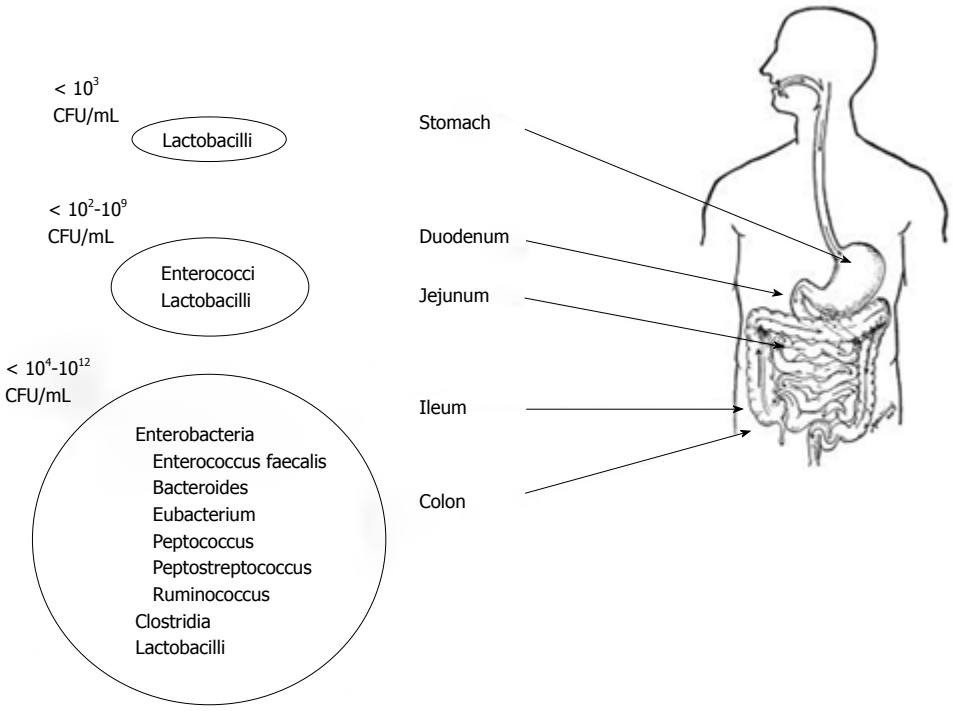

Figure 1 Bacterial composition and density along the proximal to distal segments of the gastrointestinal tract.

samples of other subjects participating the same study. While the most abundant phyla in the *H. pylori*-free stomachs were Actinobacteria, Firmicutes, and Bacteroidetes, the majority of the phylotypes that were not also found in throat samples, were identified as Proteobacteria.

In 2006, Bik *et al*[21] using a 16S rRNA clone library, described a large bacterial diversity in gastric biopsies from 23 esophagogastroduodenoscopies. Over 50% of the identified bacteria were classified as uncultivated, 10% of the phylotypes were previously uncharacterized. Overall, five major phyla (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria) composed the bacterial diversity of the stomach. The authors reported results significantly different from those obtained in studies performed on the oral cavity and esophagus and this evidence allowed them to hypothesize a separate microbial ecosystem in the stomach. Interestingly, in that study the presence of *H. pylori*, gastric anatomical location and gastric pH value had no significant effects on the composition of the gastric bacterial population.

As the pathogen microbes concerns, the hostile environment of the human stomach can be colonized by other bacterial strains able to survive in those harsh conditions and other *Helicobacter* species with typical spiral morphology have also been found to be associated with gastric lesions and cancer. The percentage of patients infected with these "non-*H. pylori* helicobacters" (NHPH), previously denominated "*Helicobacter heilmanni*", is much lower than for *H. pylori*, varying between 0.2% and 6%[22]. Several studies have investigated the association between NHPH and human disease, including Crohn's disease, lithiasis, liver disease, coronary disease, gastritis, and pyoderma gangrenosum-like ulcers[23]. In addition to NHPH, other pathogen bacteria in the stomach may influence *H. pylori*-associated gastric pathogenesis by creating reactive oxygen and nitrogen species and modulating inflammatory responses. Non-*H. pylori* bacteria and their by-products may represent a persistent antigenic stimulus, thus increasing the inflammatory response induced by *H. pylori* infection[24].

A recent study by Hu *et al*[25] described a high prevalence (65.0%) of the non-*H. pylori* bacterial flora in the *H. pylori* positive patients, which indicated that the upper GI disorders may enable non-*Helicobacter* bacteria to survive and colonize the human stomach. The majority of these species were *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, differing in composition compared to *H. pylori* non infected patients. Considering that not all the bacterial species can be cultivated due to the harsh culture condition, the number of these non-*H. pylori* pathogenic bacteria may be higher than that found.

GASTRIC CANCER

GC represents the second cause of cancer-related death worldwide, accounting for nearly 11% of cancers in males and 7% in females[26]. The GC incidence varies between populations with huge fluctuations. In Japan, the incidence is 80 per 100000 males, while in Africa the overall incidence is only 5 per 100000. In Europe, the incidence rates range from 20 to 40 per 100000[26]. In Italy, GC represents the fourth most common cancer (following lung, breast, and colorectal cancer) and its incidence yields its peak during the seven decade of life, being rare in patients younger than 40 years. Besides, the mortality in Northern and Centre regions is twofold higher than

that in Southern Italy[27].

The development of GC is a multi-step process (Correa's hypothesis) on which many individual and environmental factors contribute[28]. The most known and debated cause of GC is the presence of *H. pylori* infection. The International Agency for Research on Cancer classified *H. pylori* as a Group 1 human carcinogen. This is still the most important single risk factor for GC present in almost 50% of world population[29] and causally correlated with chronic gastritis, peptic ulcer, gastric atrophy and gastric lymphoma other than GC[30,31].

Evidences supporting the pivotal role of *H. pylori* infection in the gastric neoplastic transformation derive from studies on infected patients with mucosa associated lymphoid tissue (MALT)-lymphoma gastric tumors. After successful *H. pylori* eradication, these patients healed completely and both GC and tumor promoting proliferation of lymphoid tissue disappeared[32,33].

Low socio-economic status, *H. pylori* infection in family members living in crowded house-shelters and poor sanitation are the most favorable conditions for *H. pylori* transmission from person to person and cancer risk. In the last years, the overall GC incidence is constantly falling, possibly due to the fall in the *H. pylori* prevalence caused by increasing living standard level, but not in populations at high risk. Besides, the incidence of GC is decreasing in the distal stomach and increasing in the gastro esophageal junction[34].

Other environmental and dietary factors, tobacco use, achlorhidria and bacterial overgrowth can promote in gastric mucosa the pathways of cellular growth altering the growth/apoptosis balance[35]. The major dietary factors considered in relation to GC onset are salt ingestion, smoked foods, and frequent use of cooking oil. Previous studies showed that diets rich in these components significantly increase by two-fold the GC risk[36-38]. A possible explanation is that salt may damage the protective mucosal layer of the stomach. Besides, diets with high-salt content contribute to expansion of *H. pylori* colonization[39] and deep-oil-fried foods have been demonstrated to produce human carcinogens such as benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene, and heterocyclic aromatic amines, due to high frying temperatures[40]. By opposite, a diet with fresh foods rich in vitamin C (including fresh foods, vegetables, and fruit) has been demonstrated to be protective. An antineoplastic role for vitamin C has been hypothesized[41] since this vitamin may prevent GC cell growth, probably by blocking the formation of human carcinogenic *N*-nitroso compounds, and significantly reducing the nitrite concentration by 43%[42]. Additionally, diets with a high content in green tea[43] or other substances with postulate antineoplastic activities such as whole grain cereals, garlic or vitamins, have also been associated with a reduced risk of this cancer[44], even if there is still uncertainty if changing diet habits to introduce more of the above cited compounds would effectively reduce the GC risk[45].

POTENTIAL OF PROBIOTICS TO PREVENT NEOPLASTIC TRANSFORMATION OF GASTRIC MUCOSA

It is estimated that 20% of malignancies worldwide can be attributed to infections and the classical example is provided by the association between *H. pylori* with both GC and MALT. More in general, the chronic alteration of intestinal microbiota homeostasis could promote many diseases, including cancer. The mechanisms by which bacteria may induce carcinogenesis include chronic inflammation, immune evasion, and immune suppression. Elevated numbers of T regulatory cells have been demonstrated to suppress the innate and adaptive immune responses, thereby contributing to tumor progression[46].

The complex interactions between diet, normal intestinal microbiota and health encouraged the development of strategies that allow for the selective growth of probiotics[47]. However, although *in vitro* and animal model studies suggest a protective anticancer effect of probiotics, the results of studies performed in humans are still controversial.

Therapeutically, there has been an increased interest on the use of probiotics for prevention and treatment of a number of GI disorders, including irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), pathogenic bacterial or viral infections, antibiotic associated diarrhea, and also as antineoplastic agents in colon[48,49]. By opposite, little attention has been paid to probiotics and GC. Theorically, probiotics might exert their action also in the gastric mucosa. In the normal stomach, LAB concentrations may vary between 0 and 1000/mL. Being acid resistant, they persist in the stomach longer than other bacteria: dietary strains of bifidobacteria and lactobacilli survive in high proportions (> 80%) in the gastric environment for periods of 2 h[50]. The majority of available data on their putative mechanism of action in the stomach derives from studies on the effects against the carcinogen *H. pylori* infection[51], but the etiological mechanisms are still far from being fully understood. In fact, the acidic gastric environment represents an effective barrier against many of the pathogen bacteria that reach the GI tract.

A change of the physiological conditions of the stomach, as it occurs in different diseases (corpus atrophy or GC itself) or during drug administration [*e.g.*, long term proton pump inhibitor (PPI) therapies], may provide a chance for extraneous microbes to colonize the stomach. Predominant gastritis of the corpus leads to atrophic gastritis with a low production of gastric acid (hypochlorhydria)[52]. The inhibition of normal gastric acid secretion is considered at increased risk of developing GC[53], since it bears important side effects. The most important is the possible significant reduction of the "gastric barrier effect" and the bacterial overgrowth in the stomach and duodenum with a concentration of > 100000 viable cells/mL. As a major consequence of

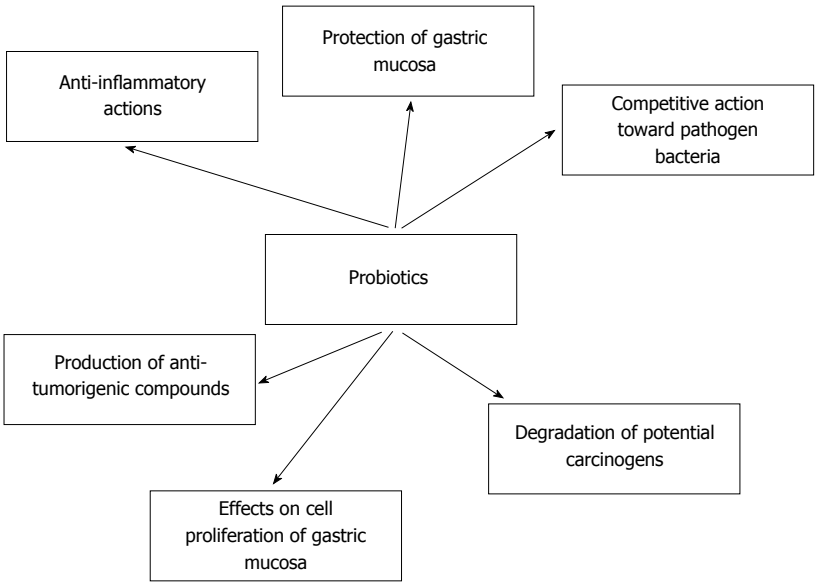

Figure 2 Proposed mechanisms of probiotic activities against gastric cancer.

Table 1 Possible mechanisms of protection of the gastric mucosal barrier induced by probiotics

Increased levels of basal mucosal prostaglandins
Increased cell proliferation/apoptosis ratio and stimulation of angiogenesis
Stimulation of local immune responses
Release of antioxidant substances
Stimulation of the expression of gastric mucins
Improvement in gastrointestinal permeability
Decreases in bacterial overgrowth

this condition, many harmful or even pathogenic bacteria contained in some foods could survive the gastric transit and colonize the stomach itself, the duodenum, or the gut, where they could establish acute and chronic infections. Some of these bacteria can reduce nitrogen and produce carcinogenic *N*-nitroso compounds through conversion of nitrates or nitrites from the saliva[54].

The possible mechanisms by which probiotics exert their effects against neoplastic transformation of the gastric mucosa concern: (1) the protection of mucosa and stabilization of the GI barrier function; (2) a competitive action toward pathogen bacteria; (3) degradation of potential carcinogens; (4) anti-inflammatory action; (5) production of anti-tumorigenic or anti-mutagenic compounds; and (6) the effects on cell proliferation and the polyamine metabolism of the gastric mucosa (Figure 2).

Protection of gastric mucosa and stabilization of the GI barrier function

Homeostasis in the stomach environment is maintained by the balance of protective and aggressive factors and drugs. An overload of aggressive factors (*e.g.*, gastric acid, stress, and alcohol) that upsets this balance can induce gastric injury.

The gastroduodenal microbiota, is low numerically, but could participate in the protection of the mucosa. Many factors may explain this protective activity and are summarized in Table 1.

It has been suggested that the protective action of probiotics on induced gastric mucosal lesions could be attributed to different factors such as prostaglandins, growth factors and cytokines. Also the regulation of cellular apoptosis, proliferation, gastric mucin production and GI permeability appears to be actively involved.

Increased levels of 6-ketoprostaglandin F1-α, epidermal growth factor (EGF) and b-fibroblast growth factor have been implicated in the protective effect displayed by peculiar bifidobacterial strains such as *Bifidobacterium brevis* (*B. brevis*)and *B. bifidum* against gastric ulceration induced by acetic acid or ethanol in rats[55]. Pre-treatment of rats with *Lactobacillus rhamnosus* GG (*L.* GG) at 10^9 CFU/mL twice daily for three consecutive days was able to markedly lessen the suppressive actions of ethanol on mucus-secreting layer and trans-mucosal resistance along with an increase in the basal mucosal prostaglandin E2 (PGE2) levels and a concomitant reduction of cellular apoptosis[56]. In a more recent study aimed at determining the role of viable lactobacilli in the healing of acetic acid-induced chronic gastric ulcer, Uchida *et al*[57] reported that a yogurt containing *L. gasseri* OLL 2716 inhibits the formation of HCl-induced acute gastric lesions through the generation of PGE2. Overall, these findings suggested that the up-regulation of prostaglandins could stimulate the mucus secretion and increase the transmucosal resistance in the gastric mucosa.

L. GG was also proven to enhance the healing of acetic acid-induced gastric ulcer in rats, *via* the attenuation of cell apoptosis to cell proliferation ratio accompanied by a significant ornithine decarboxylase (ODC) up-regulation and B-cell lymphoma 2 protein expression at the ulcer margin[58]. The phosphorylation level of EGF receptor was also up-regulated without altering the total EGF receptor expression. Angiogenesis was also signifi-

cantly stimulated together with the induction of vascular endothelial growth factor (VEGF) expression.

The role of VEGF was also investigated in a study testing the ability of the probiotic mixture VSL#3 (a combination of eight probiotic bacteria including *Lactobacilli*, *Bifidobacteria* and *Streptococcus* species) in healing acetic acid induced gastric ulcer in rats[59]. VSL#3 was administered orally at low or high dosages from day 3 after ulcer induction for two weeks. The gene expression of several pro-inflammatory cytokines, protein and expression of stomach mucin5ac (muc5ac), regulatory cytokine-IL-10, COX-2 and various growth factors were evaluated. Remarkably, the probiotic efficacy was effective at higher concentrations of VSL#3 by specifically increasing the expression and production of angiogenesis promoting growth factors, primarily VEGF that was dramatically increased after 7 d of treatment.

In another work performed on laboratory animals, Senol *et al*[60] demonstrated that the pretreatment with a probiotic mixture (13 different bacteria) attenuated the aspirin-induced gastric lesions by reducing the proinflammatory cytokines and the lipid peroxidation, enhancing mucosal sIgA production and stabilizing mucosal mast cell degranulation into the gastric mucosa.

Stimulation of the expression of gastric mucins appeared to be pivotal in the protective effects by probiotics against gastric injury. Gomi *et al*[61] investigated the properties of *B. bifidum* BF-1 in a rat model of acid-ethanol-induced acute gastric ulcer. The gastric lesions were proven to be significantly lower in the BF-1 group than in the control group, which showed a similar level to the group treated with another bacterium, the *Streptococcus thermophilus* YIT 2021 ST. The production of gastric mucin and the expression of several target genes associated with protection and inflammation were examined before and after induction of gastric injury. Muc5ac gene expression in gastric corpus samples and gastric mucin production in stomach samples from the BF-1 group were significantly higher than those in the respective samples from the control group. These findings indicated that BF-1 has the potential to provide gastro-protection, alleviating acute gastric injury by enhancing the production of gastric mucin in rats.

The importance of gastric microbiota in the protection against gastric lesions has been suggested by studies demonstrating that the alteration of homeostasis of the resident microbiota contributed to the development and persistence of injuries[62].

PPIs are commonly used to treat acid-related diseases, mainly gastroesophageal reflux disease. However, PPIs may indirectly affect the microenvironment of the flora *via* changes in pH[63]. Chronic PPI administration exposes the subject to the risk of exogenous infections as most pathogens are able to survive the gastric transit in a condition of significantly decreased acidity. In order to evaluate the ability of probiotics in preventing the "gastric barrier effect" from prolonged use of PPIs, different probiotic bacteria considered as having a strong inhibitory activity on gram-negative bacteria [*L. rhamnosus* LR06 (DSM 21981), *L. pentosus* LPS01 (DSM 21980), *L. plantarum* LP01 (LMG P-21021), and *L. delbrueckii* subsp. delbrueckii LDD01 (DSM 22106)], were tested on a group of 30 patients treated with PPIs for either short (3 to 12 mo) or long-terms (> 12 consecutive months) in comparison to a control group[64]. The total viable cells and total lactobacilli were quantified in gastric juice and duodenal brushing material from all subjects. As expected, the results confirmed the strong bacterial overgrowth in the stomach and duodenum of people treated with PPIs compared with subjects with a normal intragastric acidity. In addition, the bacterial cell counts in subjects who underwent a long-term treatment with a PPI were greater than those from subjects taking these drugs for short term. Total lactobacilli represented the major percentage of bacterial counts, thus demonstrating the ability of such bacteria to colonize the stomach and the duodenum, at least temporarily, and to consequently restore the gastric barrier effect.

Alterations in GI permeability are considered as an initial step in the development of gastric lesions such as ulcers. In a human study, Gotteland *et al*[65] demonstrated that intake of *L.* GG protects the gastric mucosa in healthy volunteers against alterations of permeability induced by the acute administration of non-steroidal anti-inflammatory drugs (NSAIDs), an important cause of gastroduodenal ulcer.

In a manner similar to that of *L.* GG, *L. gasseri* OLL2716 proved to protect the gastric mucosal permeability against aspirin using[66]. The urinary sucrose excretion (USE) test was carried out in 29 volunteers before and after probiotic treatment for 4 wk and 37 patients undergoing low-dose aspirin therapy who took *L. gasseri* OLL2716 for 16 wk. The authors found that in healthy volunteers the elevation in the USE value after aspirin loading significantly decreased after probiotic treatment. Interestingly, also in patients assuming aspirin, the USE value significantly decreased in the period with *L. gasseri* OLL2716 administration, while no significant difference was found in the period without probiotic.

Competitive action toward pathogen bacteria

Ability by probiotics to adhere to GI mucus is of considerable importance to exert a modulatory effect *in situ*, especially on the gastric mucosa surface. Probiotics can compete with and prevent establishment of pathogenic bacteria by competitive exclusion. Several studies that characterized different LAB strains have shown their ability to adhere to gastric epithelial cells *in vitro* and *in vivo*[67-69]. A wide selection of proteinaceous and nonproteinaceous surface components (lipoteichoic acids and specific structures such as external appendages covered by lectins)[70] have been demonstrated to be involved in adhesion to epithelial cells through different mechanisms, including hydrophobic and electrostatic interactions as well as passive or steric forces. In a recent paper, Chen *et al*[71] demonstrated the antagonistic activities of *L. gasseri*

Chen and *L. plantarum* 18 against *H. pylori* growth and infection. *H. pylori* adhesion to the human gastric epithelial cells SGC7901 was efficaciously inhibited by administration of the cell free supernatants and both live and dead lactobacilli. The growth of *H. pylori* in the gastric mucosa was also significantly inhibited by the administration of *L. johnsonii* MH-68 and *L. salivarius* ssp. salicinius AP-32, either alone or in combination[72].

Cui *et al*[73] isolated *L. fermenti* and *L. acidophilus* from human gastric mucosa and screened their potential anti-*H. pylori* activity and antinflammatory effects on a mouse model of *H. pylori*-associated Balb/c gastritis. The authors reported that these probiotic strains are able to adhere to gastric epithelium, exerting also a significant anti-*H. pylori* activity. Interestingly and with possible therapeutical implications, *L. fermenti* displayed *in vivo* a valid anti-*H. pylori* activity whose efficacy was almost similar to the standard triple therapy, thus significantly improving the *H. pylori* associated Balb/c gastritis.

Degradation of potential carcinogens

Among the functional properties characterizing probiotic strains, antigenotoxicity and antimutagenicity could be pivotal against cancer[74]. As demonstrated by epidemiological researches, LAB intake is related to a reduced GI neoplasm incidence[75] and experimental evidences have highlighted the ability of lactobacilli and bifidobacteria to inhibit carcinogen-induced tumor development, at least in the large intestine[76,77].

The theory of the "*luminal steady state*" suggests that the end products of carbohydrate metabolism in the intestine such as short chain fatty acids (SCFA), formic, acetic, propionic, butyric and lactic acids, may have a beneficial effect because they can act as colonic nutrients. These actions may be counteracted by the products deriving from the metabolism of proteins (phenolic compounds, amines, ammonia, *N*-nitroso compounds, and indoles) which might have detrimental effects on the host[78].

Heterocyclic aromatic amines (HCA) are compounds with high mutagenic potential, formed during the cooking of meats at high temperatures of 150-300 ℃. Numerous studies have shown that these amines are involved in the etiology of gastric and colon cancer in humans. The inactivation of HCA by LAB has been reported in several articles and studies performed *in vitro* and laboratory animals have demonstrated that probiotic bacteria prevent induction of DNA-damage and preneoplastic lesions induced by certain HCA[79,80].

Stidl *et al*[81] conducted a comprehensive investigation on the ability in inactivating HCA by different LAB species present in fermented foods or derived from the human GI tract. In their paper, the authors proved the existence of clear differences in the binding behavior of LAB towards structurally different amines. Different physicochemical properties of the amines such as the pKa-values, solubility and lipophilicity may affect their elimination by LAB. Besides and in agreement with earlier investigations[82], the experiments on bacterial mutagenicity showed that the inactivation of the amines by LAB induces a decrease of their mutagenic potential, correlating also with the binding capacities of the individual probiotic strains.

Antinflammatory action

Probiotics significantly affect the innate as well as the acquired immune system by different products such as dead cells, metabolites, parts of the cell wall, and DNA. All these components are recognized by host cells equipped with appropriated receptors. In this context, the main target is represented by epithelial and gut-associated immune cells.

A signaling cascade leading to immune modulation may be triggered by the adhesion of probiotics or their components to epithelial cells as well as the release of soluble factors. These probiotic actions could be important for the suppression of neoplastic host cells[83].

Also in this case, the main trigger for eliciting the immune cells in the stomach is represented by *H. pylori* infection. One of the well-known carcinogenesis pathways related to *H. pylori* is through the sequence "inflammation-atrophy-intestinal metaplasia-dysplasia-adenocarcinoma"[28].

The efficacy of probiotics in the treatment of inflammation-based GI diseases is both founded on the inhibition of pathogenic bacteria and inflammatory processes[84].

Lee *et al*[85] found that the enhancement of antinflammatory signals might be essential mechanisms of probiotics rather than attenuating inflammatory signals imposed by *H. pylori* infection. In their study performed on AGS cell line, the authors found that *H. pylori* or its lipopolysaccharide stimulation were able to significant increase expressions of different inflammatory mediators (*e.g.,* TNF-α, IL-8, inducible NOS and COX-2). Pretreatment of cells with *L. plantarum*, *L. rhamnosus* and *L. acidophilus* significantly inhibited these mediators. The authors postulated that these antinflammatory effects were obtained by enhancing the expression and signaling of suppressor of cytokine signaling 2 and 3 (SOCS-2 and SOCS-3). Their expression is mediated through both significant phosphorylation of signal transducers and activation of transcription (STAT)-1 and STAT-3, and simultaneous inhibition of Janus kinase (JAK)2 phosphorylation, which is known to signal SOCS-2/SOCS-3 negatively[86,87].

H. pylori can induce TNF-α and IL-8 pro-inflammatory cytokine expressions in MKN45 cells. Pretreatment with *L. acidophilus* counteracted *H. pylori*-induced IL-8 expressions, specifically by mediation through the IκBα/NF-κB pathway in a dose-dependent manner. Moreover, *L. acidophilus* ameliorated IFN-γ-induced Smad7 translation level and improved the *H. pylori*-induced gastric inflammation *in vitro*[88]. Besides, in "*in vivo*" studies, the *H. pylori*-infected gastric mucosa showed up-regulated NF-κB pathway and Th1 type cytokine responses[89,90], which may disturb the integrity of the gut epithelial barrier.

Administration of *L. rhamnosus* R0011 and *L. acidophilus* R0052 after infection eradicated *H. pylori* and reversed gastric inflammation in laboratory animals[91]. Similar results were reported with *L. gasseri* OLL2716[92].

Production of anti-tumorigenic or anti-mutagenic compounds

The effect of probiotics can be due to the production of different antimicrobial compounds and the two main categories are SCFAs and bacteriocins.

SCFAs are produced during the metabolism of carbohydrates by probiotics and have an important role in physiological functions. Since they derive from carbohydrates, these anions may be modulated by the dietary modifications. SCFAs were shown to play a key role in modulation of the digestive epithelial cells proliferation/apoptosis balance. This involves stimulation of differentiation in healthy cells, nuclear receptor activity through mitogen-activated protein kinase activation, histone deacetylase inhibition and apoptosis stimulation in transformed cells[93].

Apoptosis is a natural physiological process that regulates the number of cells and represents an ideal target for anti-neoplastic strategies. Through a series of regulated processes, cancer cells become fragmented and their residual portions are absorbed by adjacent tissues and immune system[94].

In human gastric carcinoma cell lines, both butyrate and propionate have been found to be able to induce apoptosis[95]. Two main apoptotic pathways are involved: the extrinsic pathway, mediated by activation of caspase 8 and death receptors and the intrinsic pathway, in which a role is played by caspase 9 and mitochondria[85].

It is postulated that cancer cells generally lack for apoptotic control, and hence their proliferation is constantly fostered. Therefore, the ability to induce apoptosis of cancer cells shown by different LAB may reflect an intrinsic anti-neoplastic potential. In this connection, dairy propionibacteria have been proven to produce beneficial pro-apoptotic SCFAs, hence they may be useful in cancer prevention or treatment[96].

SCFAs may also act against *H. pylori* infection. Bhatia *et al*[97] were the first to report an antagonistic action of *L. acidophilus* against *H. pylori* and to hypothesize a role for SCFAs in this effect. The reported antimicrobial activity could be due not only to a direct competition with *H. pylori*, but also to the inhibition of its urease activity, as shown with two high lactic acid producers, namely *L. salivarius* and *L. casei Shirota*[98,99].

The establishment in the stomach mucosa by probiotics is hampered by hostile conditions. Thus, administration of positive bacterial strains in combination with prebiotics (synbiotic) may facilitate their settlement and enhance their properties. Prebiotics are defined as non-digestible food ingredient able to selectively stimulate their growth and/or activity[100] and different papers have highlighted this ability, at least in the colonic lumen. Use of probiotic bacteria with oligosaccharides could promote bacterial growth and increase great quantities of butyrate, which has been shown to have antitumor effects at the cell level[48]. Rodent studies demonstrated that a synbiotic combination of resistant starch and *B. lactis* exerted a pro-apoptotic action in response to the carcinogen azoxymethane[101].

Another class of substances with a reported antimicrobial and antineoplastic activity is represented by bacteriocins. These small, heat resistant and dialyzable peptidic structures possess a spectrum of antimicrobial activity against closely related Gram positive and Gram negative pathogens, including food-borne pathogens[102].

Not all the bacteriocins show the same inhibitory activity. *L. salivarius* UCC118 produces a peptide that inhibits different pathogens such as *Bacillus*, *Staphylococcus*, *Enterococcus*, *Listeria*, and *Salmonella* species[103]. Lacticin 3147, a bacteriocin produced by a *Lactococcus lactis* strain, has shown the ability to inhibit a range of genetically distinct *Clostridium difficile* isolated from healthy subjects as well as patients with IBD[104].

Different lactobacilli have been demonstrated to produce these peptidic structures in the stomach. However, other probiotic strains such as *Enteroccus faecium*[105], *Bacillus subtilis*[106] and *Bifidobacterium*[107] could also produce heat-stable proteinaceous compounds capable of inhibiting *H. pylori* growth.

LAB strains can also produce a wide range of substances other than bacteriocins acting in a positive fashion for the gastric environment. Among them, the exopolysaccharides, polymeric substances that promote the colonization of probiotic bacteria by cell to cell interactions in the alimentary tract. Additionally, biosurfactants have shown to reduce adhesion of pathogens into gastric wall membrane due to their antiadhesive properties. LAB strains have also been reported for production of antioxidants that scavenge the free radicals such as superoxide anions and hydroxyl radicals[108].

As concerns the suggested antioxidant activities, a possible therapeutic use of synbiotic was investigated by Singh *et al*[109]. The authors evaluated the effects of a novel combination of *L. acidophilus* together with ginger extract, a bioactive phytochemical with antioxidant and antiulcer effects. This synbiotic formulation was administered to rats in a form of floating beads and demonstrated to reduce the oxidative stress and be effective in terms of ulcer index, mucus secretion, and histopathological parameters in comparison to control animals. This approach could be interesting for the management of gastric diseases since the use of probiotics is limited due to their transience and inability to survive the adverse physiological conditions of the GI tract. Therefore, packaging probiotics and prebiotics in a suitably pharmaceutical formulation may facilitate their establishment in the stomach mucosa.

Effects on the polyamine metabolism and cell proliferation of the gastric mucosa

Beyond the production of specific bacterial enzymes,

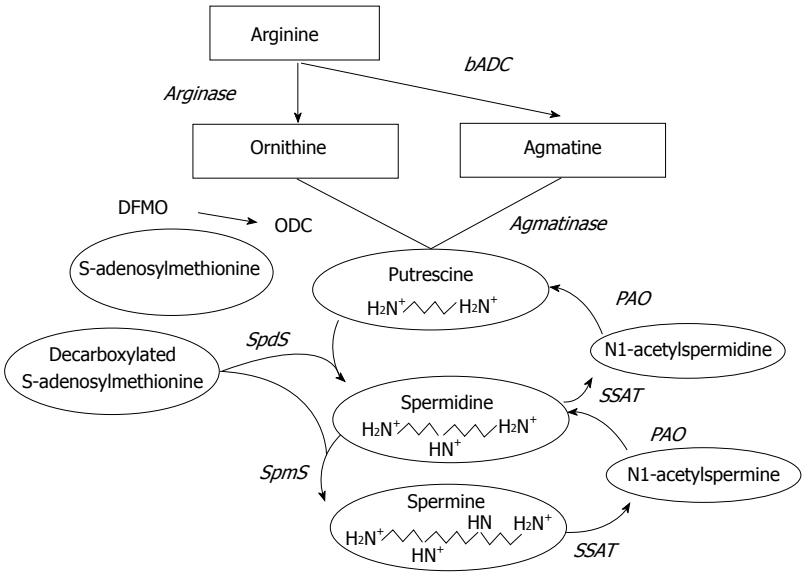

Figure 3 Schematic depiction of the polyamine metabolism. ODC: Ornithine decarboxylase; PAO: Polyamine oxidase; SSAT: Spermidine-spermine-*N*-acetyl transferase.

reactive oxygen species, and effects on the host metabolome, the mechanisms by which probiotics inhibit cancer development may involve multiple pathways, including the regulation of cell proliferation and the apoptotic processes[110]. Such anti-carcinogenic properties have also been studied at a molecular level[111] and by analysis of intermediate biomarkers of proliferation such as polyamines (putrescine, spermidine and spermine)[112]. These small molecular weight amines are highly required in both pre-neoplastic and neoplastic tissue to sustain the enhanced cell growth[113].

Polyamine synthesis is an early event occurring during the G1 phase of the cell cycle and represents a requisite for proliferating cells to start their processes. Their metabolism begins with the intervention of the rate limiting enzyme ODC that is finely tuned in all of the proliferating cells and is subjected to different growth promoting stimuli[114].

Figure 3 shows a schematic representation of the polyamine metabolic pathway. Briefly, biosynthesis is mediated by the key enzyme ODC which converts the amino acid ornithine into putrescine. This is then sequentially converted into spermidine and spermine through the action of the enzyme S-adenosylmethionine decarboxylase and spermidine/spermine synthase. The central enzyme in the polyamine catabolic pathway is the spermidine-spermine-*N*-acetyl transferase, which adds acetyl groups to terminal amine groups in spermidine and spermine. These acetylated polyamines are then substrates for the enzyme polyamine oxidase (PAO) which retro-converts these acetylated derivatives into lower chain amines.

Polyamine concentrations increase during carcinogenesis and an increase in ODC activity accompanies neoplastic transformation of the mucosa[115]. As with other tumors, the content of polyamines in GC is higher compared to the adjacent mucosa and equivalent normal tissue[116]. In the stomach, polyamines are also increased in pre-neoplastic conditions such as during *H. pylori* infection. Polyamines were evaluated in antral and body biopsies of 26 dyspeptic patients (20 *H. pylori* positive and 6 *H. pylori* negative) undergoing gastroscopy[117]. Antral and body biopsies from infected patients contained higher polyamine levels than those from non-infected subjects. In *H. pylori* positive patients, the baseline polyamine levels were higher in the antrum than in the body, whereas levels in the two stomach regions were similar in *H. pylori* negative subjects. After therapy, polyamine levels decreased in patients with successful eradication, but they remained unchanged in patients in whom infection persisted.

The increase in the polyamine content seems to be due to the loss in polyamine homeostasis occurring during the dysregulation of cell proliferation. This is also proven by evidences of an up-regulation of polyamine biosynthesis, a decrease in their catabolism, and an increased uptake[115].

Since polyamines and their enzymes are strongly related to neoplastic proliferation in the GI tract, all the strategies of interventions targeting polyamines, including probiotics, deserve deep investigations.

Our group[118,119] found that *L.* GG administration induced a significant reduction in polyamine biosynthesis in two different human GI cancer cell lines, one originating from undifferentiated carcinoma of the stomach (HGC-27), the other from colon adenocarcinoma (DLD-1). Interestingly, when the cytoplasmic extract derived from *L.* GG homogenate was tested, the cytoplasmic extract, but not the cell wall extract, was shown to be suppressive.

In an *in vivo* study, Linsalata *et al*[120] demonstrated that the ingestion of VSL#3 reduced polyamine levels and ODC activity in colorectal mucosa of rats. Besides,

in a human study by the same group, a peculiar *L. brevis* strain CD2 demonstrated anti-proliferative biochemical features[121]. In this study, a cohort of *H. pylori*-positive dyspeptic patients randomly received high oral doses of *L. brevis* CD2 or placebo, for 3 wk before endoscopy. Before and after treatment, *H. pylori* infection was determined by urea breath test. In gastric biopsies, ODC activity and polyamine levels were evaluated by a radiometric technique and high-pressure liquid chromatography, respectively. The study demonstrated that administration of *L. brevis* CD2 alone did not eradicate *H. pylori* even if a reduction in the UBT delta values occurred, suggesting a decrease in intragastric bacterial load. Significantly, *L. brevis* CD2 induced a decrease in gastric ODC activity and polyamine levels. These data support the hypothesis that *L. brevis* CD2 treatment can decrease *H. pylori* colonization, thus reducing polyamine biosynthesis. Probably, the arginine deiminase activity following the probiotic treatment might cause arginine deficiency, preventing polyamine generation from gastric cells. This enzyme induces the catabolism of arginine and can affect the biosynthesis of polyamines[122]. In this connection, in a study performed on the human T leukemia Jurkat cell line, Di Marzio *et al*[123] demonstrated that lyophilized and sonicated preparations of *L. brevis* CD2 were able not only to cause arginine-dependent polyamine synthesis inhibition, but also to induce consequently a relevant apoptotic effect.

The rates of cell proliferation and apoptosis may determine the speed of neoplastic growth[124]. Apoptosis is frequently impaired in many human tumors and is also an important phenomenon in chemotherapy-induced tumor cell death. Therefore, the modulation of apoptosis has been hypothesized as an effective technique in the treatment of cancer[125]. As postulated in the colonic environment, the induction of apoptotic processes by gut microbiota could represent the so-called physiologic "oncologic surveillance" mechanism for the proliferative disease prevention[126], but this hypothesis waits for further testing before confirmation in the gastric environment. It has been demonstrated that the antiproliferative effect and the induction of apoptosis caused by two lactobacillus strains (*L. paracasei* IMPC2.1 and *L.* GG) were quite similar in both HGC-27 and DLD-1 cancer cell lines and they were mediated not only by live microorganisms but also nonviable ones[118].

A milk fermented by *Propionibacterium freudenreichii* was recently tested for its pro-apoptotic potential on HGT-1 human GC cells. Fermented milk supernatant induced typical features of apoptosis including chromatin condensation, formation of apoptotic bodies, caspase activation and cytochrome c release, DNA laddering, cell cycle arrest, and reactive oxygen species accumulation. Moreover, this fermented milk enhanced the cytotoxicity of camptothecin, a drug used in GC chemotherapy. This kind of functional foods may represent a useful tool as part of a preventive diet designed for GC prevention and/or as a food supplement to potentiate cancer therapeutic treatments[127].

The proliferation and cell death of KATO3 cells, a GC cell line, were examined after treatment with *L. casei* extract for various times and at various doses. *L. casei* extract inhibited the growth of KATO3 cells and induced apoptosis by inactivating NF-κB promoter activity. However, apoptosis induced by *L. casei* extract was not directly associated with the intrinsic mitochondrial pathway. Immunoblot analysis revealed that *L. casei* extract decreased the expressions of NF-κB and IκB. The reduced NF-κB levels led to a concomitant decreased phosphorylation of mTOR signaling components, such as PI3K, Akt, and p70S6 kinase[128].

Other mechanisms of action may be evoked. The anti-proliferative capabilities of probiotics may be related to their previously described ability to adhere to cells. Lee *et al*[129] found that *Bacillus polyfermenticus* SCD was strongly adherent to Caco-2 cells and inhibited the growth of colon cancer cells in a dose dependent manner.

Bacterial enzyme inhibition and anticancer activity of *B. adolescentis* SPM0212 was assessed in three human colon cancer cell lines: HT-29, SW-480, and Caco-2. Cell proliferation was inhibited in all the cancer cell lines. Besides, this probiotic strain inhibited in a dose-dependent fashion TNF-α production as well as modifications in cellular morphology. Finally, this bacterial strain was proven to hinder harmful fecal enzymes, including beta-glucuronidase, beta-glucosidase, tryptophanase, and urease[130].

CONCLUSION

There is a growing body of evidence that different microbes may contribute to gastric tumorigenesis and exogenous administration of probiotic bacteria may be of some help in preventing/contrasting neoplastic transformation of the gastric mucosa. In this context, not only dietary modifications or drugs can affect the numbers and types of microorganisms in the stomach, but microorganisms can also generate *per se* new compounds from food components, some of which can be beneficial while others may be harmful.

LAB and Bifidobacteria are the most common types of microbes used as probiotics, and are also considered as markers of stability of the normal human intestinal microbiota. Against a huge amount of data suggesting a role for probiotics in CRC prevention, few investigations are available on their effects on GC. Despite this, probiotic strains have been proven to be helpful, at least in the management of a gastric pre-neoplastic condition such as *H. pylori* infection[103]. One suggested mechanism related to probiotic therapy is that these microbes can adhere and even transiently reside in the stomach, enhance the immune response, and reduce the *H. pylori* inflammation effect on the host gastric mucosa[131]. Notwithstanding, the *in vitro* and *in vivo* data here reported suggest a role for probiotics also in controlling the rate of proliferation of neoplastic cells in human GC cell lines as well as in animal stomachs. Therefore, many of these bacteria, together with microbially generated metabolites, may have a role in

GC risk or development. Nonetheless, there are still unanswered questions. Who might benefit from dietary interventions to alter their indigenous microbe population? How does individual's genetic background influences their microbiota? What are the active metabolites of food components? Can we identify inter-individual variability in the production of these substances? How can these substances be better utilized for cancer prevention?

Only when an answer for each of these questions will be available, it will become possible to develop a more specific strategy for preventing or, at least, delaying the onset of neoplastic transformation of the gastric mucosa based on precise modifications of the composition or activities of the GI microbiota.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Ever-changing endoscopic treatment for early gastric cancer: Yesterday-today-tomorrow

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Abstract

Endoscopic resection has been an optimal treatment for selected patients with early gastric cancer (EGC) based on advances in endoscopic instruments and techniques. As endoscopic submucosal dissection (ESD) has been widely used for treatment of EGC along with expanding ESD indication, concerns have been asked to achieve curative resection for EGC while guaranteeing precise prediction of lymph node metastasis (LNM). Recently, new techniques including ESD or endoscopic full-thickness resection combined with sentinel node navigation enable minimal tumor resection and a laparoscopic lymphadenectomy in cases of EGC with high risk of LNM. This review covers the development and challenges of endoscopic treatment for EGC. Moreover, a new microscopic imaging and endoscopic techniques for precise endoscopic diagnosis and minimally invasive treatment of EGC are introduced.

Key words: Endoscopic resection; Early gastric cancer; Confocal laser endomicroscopy; Sentinel node navigation; Hybrid natural orifice transluminal endoscopic surgery

Core tip: Endoscopic treatment of early gastric cancer (EGC) has been evolved along with the expansion of ESD indication and toward the question of how to achieve accurate risk assessment of lymph node metastasis (LNM). To achieve curative endoscopic treatment, not only accurate endoscopic diagnosis but precise selection of the patient of EGC without LNM should be preceded. Recently, endomicroscopy has been introduced to provide precise microscopic visualization of histology. Moreover, sentinel node navigation surgery combined ESD and hybrid natural orifice transluminal endoscopic surgery have been reported as a new minimally invasive treatment option for the EGC patients with high risk of LNM.

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INTRODUCTION

Endoscopic resection for early gastric cancer (EGC) is widely accepted as one of the standard treatments together with surgical treatment[1]. In the 2000s, advances in endoscopic instruments were achieved and various new endoscopic techniques were developed. Still, the major obstacle to endoscopic resection for EGC has been its limitations of predicting lymph node metastasis (LNM). Therefore, approaches to the next level of endoscopic treatment have been evolved in three ways. First, how we can achieve curative endoscopic resection for EGC while guaranteeing precise prediction of LNM. Second, how we can expand a definitive indication for complete resection to secure depth of invasion and lateral margin. Last, how we can manage the patients with high risk of LNM for EGC using new endoscopic techniques. In this

review, we will keep track of the progress of endoscopic resection in two time periods and suggest the prospect of the therapeutic strategy for EGC in the future.

EXPANDING INDICATION OF ENDOSCOPIC RESECTION: 2000-2010

Endoscopic mucosal resection in the early period

In Japan, endoscopic mucosal resection (EMR) technique called the "strip biopsy" for EGC was first described in 1984, which is to lift the lesion with a grasper and to remove the lesion using a double-channel endoscope after submucosal injection of saline under the lesion. In 1988, new EMR technique, which cuts the lateral margin using needle knife (EMR-P), was introduced[2]. It helped the precise en bloc resection by resecting with a snare after peripheral cutting of the lesion. EMR was known to be a minimally invasive and safe technique and became an axis of treatment for EGC[3]. However, this technique requires skillful endoscopists and has a higher perforation risk. Then, EMR with a cap-fitted endoscope (EMR-C) for early esophageal cancer was developed in 1992 and used to treat relatively small EGC[4]. Another technique is EMR with ligation (EMR-L), which was started as a standard endoscopic variceal ligation[5]. These techniques helped to resect the lesion more safely and quickly. It also reduced the risks of perforation and bleeding during the procedure. Absolute indication of EMR for EGC was published by Japanese Gastric Cancer Association in 1998[6,7]: (1) elevated cancers less than 2 cm in diameter, and (2) small (< 1 cm) depressed cancers without ulceration. These lesions must also be differentiated cancers confined to the mucosa, and have no lymphovascular invasion. As long as the lesion was completely removed, EMR showed good long term outcome compared to surgery. The 5-year overall survival rates and recurrence rates did not differ significantly between the EMR and surgery groups (93.6% *vs* 94.2% and 1.2% *vs* 1.1%)[8]. Although the risk of metachronous gastric cancer was significantly higher in the EMR group than in the surgery group (5.8% *vs* 1.1%), most metachronous gastric cancers were successfully retreated by EMR or surgery. A major limitation of EMR was incomplete resection for lesions larger than 2 cm in diameter due to the size limitations of accessories such as snares, caps and ligating devices. Piecemeal resection also caused a high risk of local recurrence (2.3%-36.5%)[9-12]. So, the size limitations for en bloc resection of EGC kept demanding improvement in techniques.

ESD era

In the late 1990s, ESD was developed for complete removal of EGC regardless of its size and location by dissecting the submucosal layer via the use of through-the scope endoscopic knives[13]. ESD is superior to EMR because it enables en-bloc resection and precise pathologic staging for large EGCs. Now, it has become one of the standard treatments and is being used to achieve en bloc resection for EGCs that would otherwise require piecemeal or surgical resection[10,14,15]. Owing to the development of ESD technique, indications of endoscopic resection for EGC were expanded, based on the analysis of 5265 surgical specimens of EGC patients who underwent gastrectomy with D2 level lymph node dissection[16]. The recent expanded criteria are differentiated type cancers without evidence of lymphovascular invasion, including: (1) mucosal cancer without ulceration, irrespective of tumor size; (2) mucosal cancer with ulceration, less than 3 cm in diameter; and (3) minimal (500 μm from the muscularis mucosa) submucosal invasive cancer less than 3 cm in size. After ESD, the patients with EGC of expanded indication may be followed closely without surgery because they have very small risks for LNM[16-18]. However, it should be mentioned that the data provided from these observations were surgical specimens sliced at 5 mm and not 2 mm as required for ESD specimens.

Asian studies have reported that ESD has achieved a high rate of en bloc resection (89.7%-96.7%) and complete resection (84%-94.7%) (Table 1)[8,10,11,17,19-25]. Recent meta-analyses to compare the efficacy and safety of ESD and EMR for EGC showed that ESD had advantages in en bloc resection rate, histologically complete resection rate and local recurrence rate even for small lesions[26-28]. When the lesions were classified by size, the 5-year recurrence-free rate was significantly lower in the EMR group compared with the ESD group especially for the lesions larger than 10 mm. The 5-year overall survival rates and recurrence-free rates of ESD have been reported to 93.6%-100% and 98.7%-100%. For complications, delayed bleeding occurs more during ESD, with an incidence rate of up to 7%-15.6%. Perforation is higher during ESD (3.6%-4.5% *vs* 1.0%-1.2%), which was endoscopically managed in most cases. To demonstrate the efficacy and safety of ESD especially for EGCs in expanded indication, well-controlled, prospective randomized trials with a large population and long-term follow-up periods are needed.

As ESD procedure has become widespread in western countries, several small reports have shown a high success rate of en bloc resection (79%-100%) and complete resection (64.3%-100%) (Table 1)[29-36]. For complications, the rate of perforation and bleeding has been reported ranging from 0% to 8% and 0% to 8%. The long-term follow-up data are needed to evaluate the therapeutic outcome.

Challenges to ESD

To guarantee the curative endoscopic resection or stratify the risk of LNM for EGC, several key points should be checked for the pathologic diagnosis of ESD specimen[37]. Complete resection should be confirmed by the precise lateral and vertical margin status. The distance from the lateral margin of the tumor to the margin of the specimen should be described. In case of positive lateral margin, the number of sections and the extent showing positive tumor cells should be documented. If ESD specimen shows a positive vertical margin, the

Table 1 Therapeutic outcomes of endoscopic resection for early gastric cancer

Ref.	Published year country	No. (lesion/patients)	Method		En bloc resection (%)		Complete resection (%)		Follow-up (mo/range)	Complications				Recurrence rate (%)		5-yr overall survival rate (%)	5-yr recurrence-free rate (%)
										Bleeding		Perforation					
			EMR	ESD	EMR	ESD	EMR	ESD		EMR	ESD	EMR	ESD	Local	Metachronous		
Oda *et al*[15]	2006 Japan	714/655	411	303	56.0	66.3	61.1	73.6	38 (6-60)	0.1	0	1.2	3.6	7.5	NA	99.2[1]	94.4[2]
Oka *et al*[11]	2006 Japan	1020/896	825	195	42.1	83.1	23.6	83.1	EMR 83.2 ± 34.6 ESD 19.4 ± 9.2	7.6	22.6	4.8	8.7	3.1	NA	NA	NA
Imagawa *et al*[21]	2006 Japan	196/185		196		93.0		84.0	22.8 (12-46)				6.1	0.0	NA	NA	NA
Chung *et al*[19]	2009 Korea	534/1000 (dysplasia 466)		534		95.3	87.7		NA		15.6		1.2	NA	NA	NA	NA
Jang *et al*[22]	2009 Korea	198/198		198		89.7		87.9	30 (9-49)		7.4		2.9	5.1	NA	NA	94.9[2]
Min *et al*[24]	2009 Korea	346/243	103	243	77.7	95.9	75.7	88.9	29 (4-44)	3.9	5.3	1.9	4.5	0.0	6	NA	NA
Isomoto *et al*[25]	2009 Japan	589/551		589		94.9		94.7	30 (6-89)		1.8		4.5	0.0	NA	97.1	100[3]
Goto *et al*[20]	2009 Japan	276/231		276		96.7		91.7	36 (2-39)		5.1		4.0	0.9	NA	96.2	100[3]
Nakamoto *et al*[10]	2009 Japan	202/177	80	122	53.8	94.3	37.5	92.6	54 (12-89)	0		1.6		2.5	NA	100.0	100
Choi *et al*[8]	2011 Korea	215/215	215				71.2		81 (56-94)					NA	NA	93.6	98.7
Ahn *et al*[17]	2011 Korea	1370/1244	Absolute Ix						32 (22-48)								98.8-99.0[2]
			355	182	72.4	65.9	94.4	83.0		1.4	0.8	0.3	1.2	1.5 0.5	6.1 2.2	95.8-95.3	
			Expanded Ix														
			497	336	96.8	95.5	97.8	91.1		1.6	2.1	1.6	2.4	1.6 0.8	13.7 4.0	96.8	98.52
Catalano *et al*[29]	2009 Italy	48/45	36	12	72.0	92.0	56.0	92.0	31 (12-71)	8.0	8	0.0	8.0	0.0	NA	NA	NA
Dinis-Ribeiro *et al*[30]	2009 Portugal	19/19		19		79.0		89.0	10		5		0.0	0.0	NA	NA	NA
Probst *et al*[34]	2010 Germany	91/83 (EGC 66)	1	85	100.0	88.2	90.0	68.6	27 (1-71)	3.5		1.2		6.6	NA	NA	90.1[2]
Schumacher *et al*[36]	2012 Germany	30/30 (EGC 21)		30		90.0		64.3	22		4		6.0	10.7	NA	NA	NA
Repici *et al*[35]	2013 Italy	42/42 (EGC 10)		42		100.0		92.8	19 (9-53)		7.1		0.0	5.0	NA	NA	NA
Chaves *et al*[32]	2013 Brazil	62/61 (EGC 55)		62		82.2		77.4	11.3 (1-30)		0		4.8	0.0	3.5	NA	NA

[1]3-year overall survival rate; [2]3-year recurrence free rate; [3]5-year disease specific survival. EGC: Early gastric cancer; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; NA: Not applicable; Ix: Indication.

positive tumor site and the distance from the lower edge of muscularis mucosae to the positive margin should be demonstrated. In addition, depth of invasion, histologic type, lymphovascular invasion of the tumor should be evaluated. If the undifferentiated type is mixed within the differentiated type cancer, the proportion of undifferentiated type should be evaluated to predict the risk of vascular invasion and LNM. In case of submucosal invasive cancer, the extent of submucosal invasion and histologic type should be described to determine additional surgery. It is important to identify the muscularis mucosae by using the immunohistochemistry of desmin, because the risk of LNM is higher when the tumor depth is 500 μm or more from the lower edge of muscularis mucosae (≥ sm2) than sm1. For careful microscopic examination of vascular invasion, Victoria blue staining is helpful, and immunohistochemistry of D2-40 is useful for evaluation of lymphatic invasion.

Non-curative resection or high-risk of recurrence

Noncurative resection is defined as the presence of positive lateral or vertical resection margins, submucosal and lymphovascular invasion, or undifferentiated histology, which means high-risks of recurrence or LNM. Conceptually, the patients with incomplete resection after ESD can be managed with laparoscopic gastrectomy with lymph node dissection. However, when only a small portion of positive lateral margins or unclear lateral margins are found on the post-ESD specimen, this may suggest a lower risk of LNM in the cases having no other factor of noncurative resection[38,39]. The rate of residual cancer in the positive lateral margin group (25.0%) was reported to be significantly lower than that in the positive vertical margin group (33.3%) or in the positive lateral and vertical margin group (66.7%) among the patients who underwent curative gastrectomy due to non-curative endoscopic resection for EGC[40]. The patients having mucosal cancer with lateral cut-end-positive status with no LNM can be recommended to have close follow-up or endoscopic treatment[41]. Another report demonstrated that neither residual cancer nor LNM was found in the patients with less than 500 μm submucosal invasion without margin involvement in endoscopically resected specimens among 43 patients who were operated on due to residual mucosal cancer, a mucosal cancer larger than 3 cm, or a submucosal cancer regardless of size or margin involvement[42]. Lymphatic involvement and tumor size have been reported to be independent risk factors for LNM in EGC with submucosal invasion[43,44]. Based on the results of the studies, endoscopic resection may be feasible for highly selective submucosal cancers with no lymphovascular invasion. Gastrectomy with lymph node dissection is recommended to patients with positive vertical margins, submucosal involvement having high risk features or lymphovascular invasion.

Recent issues are on whether laparoscopic lymph node dissection without gastrectomy can be performed if the resection margins are negative in the patients with high-risks of recurrence and non-curative resections based on the presence of other criteria including submucosal invasion, lymphovascular invasion, or undifferentiated adenocarcinoma. One report evaluated the efficacy of diagnostic and therapeutic laparoscopic lymph node dissection after ESD in EGC patients with high-risks of LNM including undifferentiated adenocarcinoma, submucosal cancer, immunohistochemically-positive cytoplasmic staining for vascular endothelial growth factor, lymphovascular invasion, a high lymphatic microvessel density, or high microvessel density[45]. All of the dissected lymph nodes were free of cancer cells in all 9 patients. During 16 mo of follow-up, no patients had an evidence of tumor recurrence. In a retrospective study with a small number of patients, the area for lymph node according to the location of the tumor and/or the lymphatic drainage of the stomach was visualized with standard laparoscopy or infrared-ray electronic laparoscopy after submucosal injection of indocyanine green (ICG) around post-ESD scars[46]. The study showed that 2 out of 20 (10%) patients had lymph node metastases confirmed after lymph node dissection without gastrectomy, and none had local or distant recurrence at a median follow-up of 61 mo. However, this approach cannot be generalized in clinical practice yet.

Undifferentiated adenocarcinoma

Traditionally, poorly differentiated adenocarcinomas were candidates for surgery. However, in a retrospective study, 1362 patients with EGC of signet ring cell histology who underwent gastrectomy showed the similar rate of LNM compared with the patients with differentiated EGC[47]. A recent report showed that LNM was significantly associated with female sex, tumor size, depth of tumor invasion and lymphatic involvement in poorly differentiated EGC[48]. Although endoscopic management for the patients with undifferentiated adenocarcinoma is still controversial, small studies have reported successful ESD for lesions smaller than 20 mm without lymphovascular invasion[49-51]. Another study showed that poorly differentiated EGC confined to the mucosa or with minimal submucosal infiltration (≤ 500 μm) could be considered for curative EMR due to the low risk of LNM[52]. Moreover, a study showed that EGC with signet ring cell histology can be treated by EMR, if it is smaller than 25 mm, limited to the sm2 layer, and does not involve the lymphatic-vascular structure[53]. However, larger lesions showing submucosal invasion and ulceration lower the possibility of curative resection with ESD. A recent report showed that ESD for undifferentiated EGC can achieve curative resection with an excellent 5-year mortality rate[54]. En bloc and R0 resection were achieved in 99.0% and 90.7%. Curative resection was achieved in 63.9%. Among the patients who had additional surgery, the rate of local residual tumor and LNM was 4.8% and 9.5%. None had local recurrence or lymph node or distant metastasis in the patients with curative resection during a median follow-up of 76.4 mo. Until now, it is not clear that ESD

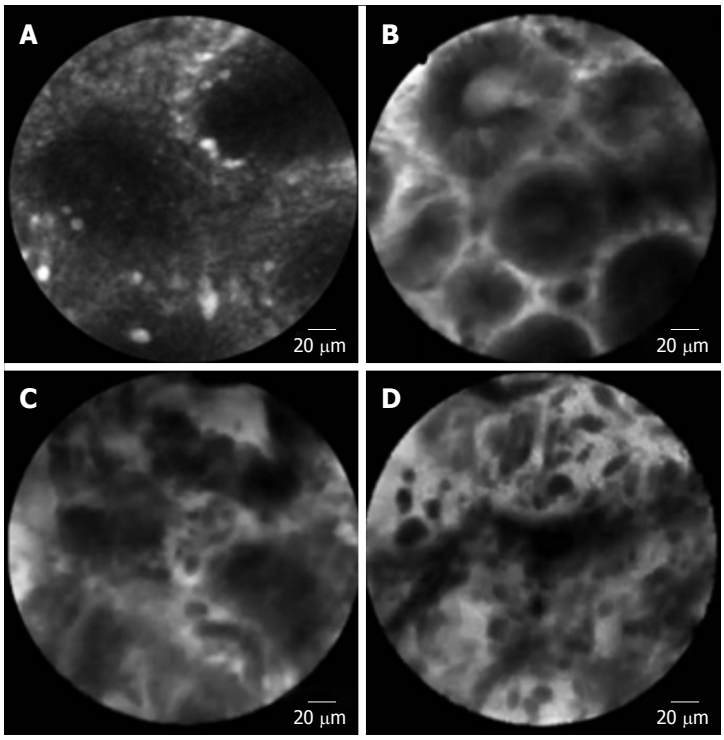

Figure 1 Features of confocal endomicroscopy. A: Normal gastric epithelium, round pattern of normal crypts is observed; B: Dysplasia, dark epithelium with irregular and varying thickness is observed; C: Differentiated adenocarcinoma, disorganized epithelium with dark and irregular glands is observed; D: Undifferentiated adenocarcinoma, dark and irregular cells with no identifiable glandular structures are observed.

is just as effective in cases of undifferentiated type EGC because the rate of curative resection is lower for undifferentiated cancer than differentiated cancer, ranging 45%-89%, in spite of high en bloc resection rate.

ADVANCES IN DIAGNOSTIC AND THERAPEUTIC ENDOSCOPY: 2010-NOW

To expand ESD criteria, instrumental and technical advances in diagnostic and therapeutic endoscopy have been challenged. Early detection of gastric cancer or precancerous lesion as well as precise staging is integral to curative endoscopic resection. Over the past decades, several advances in diagnostic endoscopy including magnifying endoscopy, narrow-band imaging, and virtual chromoendoscopy have allowed improvement in tissue characterization by detailed imaging of the mucosal pit pattern and microvascular structures. However, these techniques could not provide microscopic visualization of histology. Microscopic imaging is aimed not only to predict histology, but to visualize actual microscopic mucosal architectures in real time, high resolution and high magnification. Moreover, it is useful in microscopically guided target biopsy for EGC because it can avoid sampling errors caused by conventional biopsies in ill-defined, large mucosal cancers. Lastly, it helps to determine the margin of EGC before ESD.

Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is a system using laser light (currently blue laser light of 488 nm) for excitation and capture of laser-induced fluorescence from the defined lesion. Usually, exogenous fluorophores (intravenous fluorescein, 2.5 mL, 10%) are used to enhance the optical contrast[55-57]. There are 2 types of CLE, endoscopy-based CLE (eCLE)[58], which is integrated into an endoscope, and through-the-scope probe-based CLE (pCLE)[59] that can be inserted through the working channel of endoscopes. Compared with eCLE, pCLE shows somewhat lower resolution, but faster image acquisition. It also provides microscopic video sequences and can be used into the bile duct or through ultrasonography-guided needles. For accurate interpretation of microscopic images, adequate training in the endoscopic technique and knowledge about histopathology of EGC is required. In 2004, the first study on CLE was reported in patients who performed screening colonoscopy[55]. In the stomach, several studies have been reported CLE imaging for Helicobacter pylori infection and gastritis[60,61], intestinal metaplasia[62] and hyperplastic and adenomatous polyps[63]. From the Miami classification[64], the key features used to distinguish non-neoplastic tissue, dysplasia, and adenocarcinoma are as follows: (1) normal or non-neoplastic mucosa, round regular crypts, cobblestone appearance of normal glands; (2) dysplasia, irregular crypt lumen, dark irregular thickened epithelium; and (3) gastric adenocarcinoma, completely disorganized epithelium, fluorescein leakage, dark irregular epithelium. Differentiated and undifferentiated adenocarcinoma can be distinguished based on the presence of discriminable glandular structures[58] (Figure 1). In the studies to evaluate efficacy in pre-ESD pathologic diagnosis or post-ESD surveillance for high-grade neoplasia and superficial gastric cancer, CLE showed high accuracy (91.7%-99%) and decreased biopsies. Moreover, CLE would have directed 10% of the patients to surgery instead of ESD by correctly showing undifferentiated carcinoma[58]. CLE is

a promising technology for identifying EGC and has potential to decrease the rate of discrepancy pre- and post-ESD histopathology. The limitation of CLE is that the endoscopists who perform the *in vivo* CLE diagnosis are unavoidably biased by the endoscopic appearance of the lesion, which may have affected the in vivo CLE diagnosis. The efficacy of CLE should be confirmed in a larger population including more non-neoplastic and dysplastic lesions.

Beyond ESD

As mentioned above, a major limitation of ESD for curative treatment of EGC is inaccuracy in lymph node status. Ultimately, ESD is a curative treatment modality only if EGCs do not have regional LNM. N staging for EGC is mostly performed by CT or EUS, but diagnostic yields were not so satisfactory. EUS has a limitation not only to evaluate of regional LNM but to predict depth of invasion. It takes a lot out of the patients and endoscopists to decide and follow up after ESD. Finally, it is most important to decide what could be a minimally invasive treatment for EGC patients with a potential to escape the expanded ESD indication. Some patients who underwent surgical operation are diagnosed as mucosal cancer without LNM on the final pathology. In contrast, it is not unusual that some patients are required to have additional surgery or to give careful consideration of additional surgery after ESD. Because of these important problems, a paradigm shift has been emerged.

ESD with sentinel node navigation

Sentinel lymph node is the hypothetical first lymph node or group of nodes draining a cancer and is considered the first site of micrometastasis along the route of lymphatic drainage. Sentinel node navigation is defined as a novel, minimally invasive surgery based on sentinel node mapping and the sentinel node-targeted diagnosis of nodal metastasis. The concept of sentinel node has evolved from the surgical staging of both breast cancer and melanoma. It avoided unnecessary prophylactic radical lymphadenectomy such as axillary lymph node dissection in breast cancer patients with negative sentinel node for cancer metastasis. Although the clinical application of sentinel node mapping for EGC has been controversial for years, sentinel node mapping, using a dual-tracer method that utilizes radioactive colloids and blue dyes, is currently considered the most reliable method for the stable detection of sentinel nodes in patients with EGC[65,66]. An accumulation of radioactive colloids facilitates the identification of sentinel nodes even in resected specimens, and the blue dye is effective for intraoperative visualization of lymphatic flow, even during laparoscopic surgery. Usually, technetium-99m tin colloid, technetium-99 m sulfur colloid, and technetium-99m antimony sulfur colloid are used as radioactive tracers. Isosulfan blue, patent blue, and indocyanine green (ICG) are currently the preferred dye tracers. The patients with clinical T1N0 (< 4 cm) gastric cancer can undergo sentinel node mapping and biopsy without limitation of tumor location. Radioactive colloids and blue dyes are injected the day before surgery and just before the procedure into four quadrants of the submucosal layer around the primary tumor using an endoscopic puncture needle. Studies are investigating sentinel lymph node navigation using endoscopic injection of radiocolloid dye or ICG[65,67], or CT lymphography[68,69] using nanoscale iodized oil emulsion to increase the accuracy of detecting LNM[70]. A recent meta-analysis showed that the sentinel node detection rate, sensitivity, negative predictive value, and accuracy were 93.7%, 76.9%, 90.3%, and 90.2%, respectively[71]. When considering laparoscopic procedure, sentinel node identification rate, sensitivity, false negative rate, and accuracy were 89.3%, 68.6%, 31.4%, and 92.6%, respectively. Combined ESD and sentinel node navigation surgery might be a feasible, minimally invasive procedure that allows en bloc tumor resection to be achieved while assessing the pathological status of the regional lymph nodes (Figure 2). A case series reported that combined ESD and sentinel node navigation was conducted for 13 patients with clinical T1 N0 (≤ 3 cm) EGC, and was completed in 12 patients[72]. One patient was converted to gastrectomy after sentinel node navigation surgery. En bloc resection was achieved in all other cases.

Hybrid natural orifice transluminal endoscopic surgery

The risk of LNM in EGC exceeding the indication has known to 5.7%-20%[9]. In other words, at least 80% of patients might potentially save their stomach with curative endoscopic treatment if depth of invasion of the tumor is within the submucosa and microscopic vertical margin is secured after ESD.

Natural orifice transluminal endoscopic surgery (NOTES) may be applied as a modified treatment for EGC. NOTES means that abdominal operations are performed with an endoscope passed through a natural orifice (*e.g.*, mouth, urethra, anus) and then through an internal incision in the stomach, vagina or colon[73]. This procedure allows flexible endoscope to reach organs outside the lumen of the bowel. NOTES is minimally invasive compared to open surgery is exposed to fewer risks. Hybrid NOTES enables minimal tumor resection using the ESD technique, and laparoscopic lymphadenectomy can be performed simultaneously in cases of EGC with high risk for LNM. Hybrid NOTES for EGC means endoscopic full-thickness gastric resection (EFTGR) with laparoscopic regional lymph node dissection. It consists of endoscopic full-thickness gastric resection and laparoscopic lymphadenectomy after sentinel node navigation. EFTGR consists of five major procedures: (1) marking around the lesion safety margin confirmed by margin biopsies; (2) a circumferential incision as deep as the submucosal layer around the lesion; (3) circumferential endoscopic full-thickness resection around the lesion through the submucosal incision line under the laparoscopic guidance; (4) laparoscopic full-thickness resection around the remaining lesion through the EFTGR incision line inside

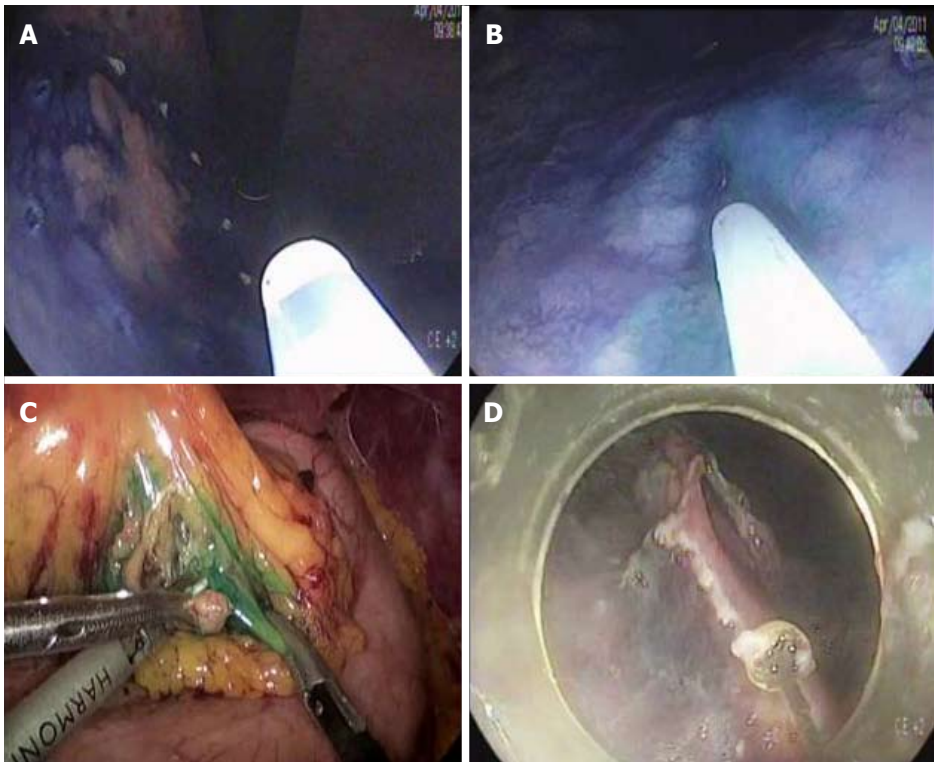

Figure 2 Endoscopic submucosal dissection with sentinel node navigation. A: Marking for endoscopic submucosal dissection is performed around the tumor; B: Indocyanine green is injected into the submucosal layer around the tumor for sentinel node navigation; C: Sentinel node harvest is performed by laparoscopic pick-up biopsy; D: Endoscopic submucosal dissection is performed.

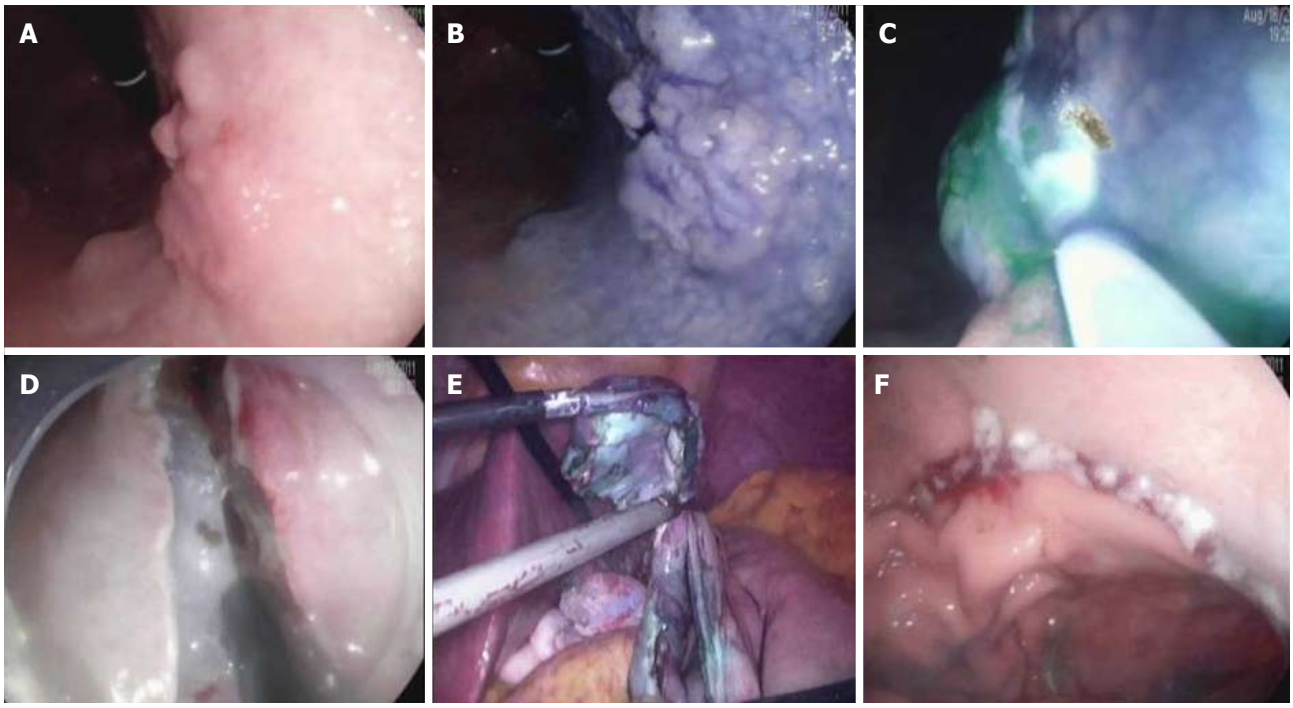

Figure 3 Full-thickness gastric resection. A: An elevated lesion is noted at the lesser curvature of upper body; B: The lesion becomes distinct by chromoendoscopy using acetic acid and indigocarmin; C: For sentinel node navigation, indocyanine green is injected into the submucosal layer after marking around the tumor; D: Endoscopic full-thickness resection is performed after sentinel node harvest and regional lymph node dissection; E: Final resection is performed with laparoscopy; F: Gastric closure is achieved with laparoscopy.

the peritoneal cavity; and (5) laparoscopic closure of the resection margin[74] (Figure 3). The lymph node dissection is performed before the full-thickness resection. Depending on the location of the lesion, the regional lymph

nodes are dissected after sentinel lymph node navigation. The first prospective, pilot study for 14 patients with EGC was published in Korea[75]. The case series concluded that hybrid NOTES could be a bridge between endoscopic resection and laparoscopic surgery and may prevent extensive gastrectomy with lymphadenectomy in patients with EGC. EFTGR has a limited indication because of the potential for tumor dissemination into the abdominal space during the procedure and vagus nerve injury. Until now, several studies have been published, and techniques are being developed to accomplish non-exposed endoscopic wall-inversion surgery[76-78]. This new method may be an alternative to surgery in patients with submucosal cancer with or without ulceration, or mucosal cancer technically difficult to resect with ESD[74,76,78].

Upcoming challenges in the new era

The key to improving therapeutic outcomes for EGC is early detection and accurate diagnosis. In spite of many advantages, endomicroscopy including CLE is still limited to some tertiary centers throughout the world. The biggest restraint in using CLE is that most health-care systems do not offer a billing code for this kind of advanced and optimized endoscopy. Clinical use of CLE before ESD will provide more accurate diagnosis of EGC compared with biopsies. Moreover, advance in endoscopic instruments, techniques and training is essential to improve outcomes of patients with EGC. Recently, novel laser system for ESD was introduced. ESD was completed using only the thulium laser, instead of endoscopy knives, without significant complications in all 10 patients[79]. In the near future, the concept of endoscopic surgery including ESD with sentinel node navigation and hybrid NOTES is expected to become one of the treatment options for the selective EGC patients with high risk of LNM.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Short- and long-term outcome of interferon therapy for chronic hepatitis B infection

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Abstract

Hepatitis B virus (HBV) infection is a serious clinical problem worldwide. Conventional interferon (IFN)-α has been approved for the treatment of chronic hepatitis B (CHB). Short-term studies have demonstrated that IFN-based therapy is moderately effective in inducing the loss of hepatitis e antigen (HBeAg) or seroconversion (30%-40%) in HBeAg-positive patients and also produces sustained HBV DNA suppression (20%-30%) in HBeAg-negative patients. Many studies have reported a correlation between the HBV genotype and response to IFN treatment. The highest response rate to IFN treatment was found in patients infected with HBV genotype A, followed by HBV genotypes B, C, and D. The long-term effect of IFN-α on CHB has not yet been elucidated. The ability of IFN-α treatment to prevent new cirrhosis, complications associated with cirrhosis, and development of hepatocellular carcinoma (HCC) is controversial. The beneficial effect of IFN-α treatment in reducing the development of HCC has mainly been observed in treatment responders who already have cirrhosis. These inconsistent findings may be attributed to the inevitable limitations of comparisons across studies, including differences in the baseline characteristics of the study and the moderate suppression of HBV replication by IFN-α relative to nucleoside/nucleos(t)ide analogs.

Key words: Chronic hepatitis B; Hepatitis B virus; Interferon α; Long-term outcome; Hepatocellular carcinoma

Core tip: The long-term ability of interferon (IFN)-α treatment of chronic hepatitis B virus (HBV) infections to prevent new cirrhosis, complications associated with cirrhosis, and development of hepatocellular carcinoma (HCC) is controversial. The beneficial effect of IFN-α treatment in reducing the development of HCC has mainly been observed in treatment responders who already have cirrhosis. These inconsistent findings may be due to the inevitable limitations of comparisons across studies, including differences in the baseline characteristics of the study and the moderate suppression of HBV replication by IFN-α relative to nucleoside/nucleotide analogs.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem that affects approximately 400 million people worldwide[1]. The clinical manifestations of HBV infection can range from acute or fulminant hepatitis to various forms of chronic infection, including chronic hepatitis,

cirrhosis, and hepatocellular carcinoma (HCC). The age of acquisition of HBV plays an important role in determining the natural history of HBV infection and is best illustrated by the differences observed between Asian and Caucasian patients. The majority of Asian patients acquire HBV via vertical transmission or early horizontal transmission within the first few years after birth, whereas the majority of Caucasian patients acquire the infection during adolescence or in early adulthood. The course of patients infected early in life is characterized by a prolonged immunotolerant phase followed by a prolonged phase of immunoclearance, typically during the third and fourth decades of life. In contrast, the majority of patients infected during adolescence or early adulthood will immediately enter the immunoclearance phase without enduring an immunotolerant phase[2]. Seventy-five percent of HBV carriers are from Asian countries, where the incidence of HCC is relatively high, at 14 to 36 per 100000 men compared to 5 to 10 per 100000 men in Europe and 2 to 5 per 100000 men in America, Australia, and New Zealand[3].

The risk of developing HCC is 200-fold higher in patients with a chronic HBV infection than in those without[4]. A large-scale cohort study reported that the risk of HCC is higher in hepatitis e antigen (HBeAg)-positive patients than in HBeAg-negative patients[5]. The most significant risk factor for hepatic carcinogenesis may be the viral load. Active HBV replication is the key driving force of disease progression, including the development of cirrhosis and HCC[6].

To date, ten HBV genotypes (A-J) have been identified[7-10] based on an intergroup divergence of 8% or more in the complete genomic sequences. HBV genotypes influence long-term outcomes in patients with chronic hepatitis B. Genotype C is associated with more severe liver disease, a faster progression of liver fibrosis, and a higher risk of HCC than is genotype B, which may be because of the higher prevalence of basal core promoter mutations[11,12]. The effects of genotypes and HBV DNA appear to be additive in the process of hepatic carcinogenesis[13,14]. HBV genotypes correlate not only with clinical outcomes but also with the response to interferon (IFN) treatment.

The primary aim of therapy is to eliminate or permanently suppress HBV, to reduce the activity of hepatitis, and to slow or limit the progression of liver disease. The ultimate long-term goals of therapy are to achieve a sustained viral response (SVR) and to clear the surface antigen (HBsAg), thus preventing or reducing the development of hepatic decompensation, cirrhosis, or HCC as well as to prolong survival[15].

Seven agents have been approved to treat chronic hepatitis B, which include two immunomodulators (conventional IFN-α and pegylated IFN-α) and five nucleoside/nucleotide (NA) analogs (lamivudine, adefovir, entecavir, telbivudine, and tenofovir). IFN and NA, which are available therapies against chronic hepatitis B that suppress the activity of HBV, work either by stimulating the immune system to eliminate virus-infected cells or to inhibit viral replication, respectively.

Prior to the recent approval of NA therapy, IFN-α was the first agent approved for chronic hepatitis B therapy. The main advantages of IFN-α over NA are the absence of resistance and the possibility of immune-mediated clearance of HBV. Unfortunately, side effects preclude the use of IFN-α in large numbers of patients, and prolonged maintenance therapy to suppress HBV is not feasible[16].

All of the studies that have previously investigated the impact of IFN-α treatment on HBV-related liver disease progression were conducted with conventional IFN-α because pegylated IFN-α was recently licensed and long-term studies have not yet to be published.

Previous studies have shown that antiviral therapy can improve both the short-term and long-term outcomes of chronic hepatitis B. However, long-term follow-up studies of IFN-α treatment for chronic HBV infection have reported conflicting findings. The ability of the treatment to prevent cirrhosis and the development of HCC remains poorly understood.

SHORT-TERM STUDIES OF IFN-α

Alanine aminotransferase (ALT) levels, HBV DNA titers, and the degree of liver inflammation have been associated with predicting the IFN response in chronic HBV infection[17,18]. Short-term studies have shown that IFN-based therapy is moderately effective in inducing the loss of HBeAg or seroconversion (30%-40%) in HBeAg-positive patients[17,19-22] as well as in producing sustained HBV DNA suppression (20%-30%) in HBeAg-negative patients[22-25].

Many studies have reported a correlation between the HBV genotype and response to IFN treatment. Table 1 summarizes the details of studies of the response to IFN-based treatment on HBV genotypes. Because most of the available information on HBV is from Asia, the United States, and Europe, the predominant genotypes of these countries, A, B, C, and D, are prominent in the literature. HBV genotype B was associated with a better response to IFN-based treatment compared to that of HBV genotype C (39% *vs* 17%, $P < 0.03$) in a Chinese study of 73 HBeAg positive patients[26]. A study from Taiwan comprising 58 HBeAg positive patients also demonstrated a better response in HBV genotype B than in HBV genotype C infection (41% *vs* 15%, $P < 0.05$)[27]. A small study, with only HBeAg negative patients treated with highly variable IFN doses of 3 × 1 MU to 3 × 8 MU weekly, suggested a better response rate in patients with HBV genotype A than with HBV genotype D or E[28]. A study from Germany involving 144 patients with HBV genotype A or D (99 HBeAg positive and 45 HBeAg negative patients) investigated the sustained response (six months after treatment) rate to standard IFN-α therapy[29]. The sustained response rate was higher in HBV genotype A- than in HBV genotype D-infected

Table 1 Summary of the studies of the response to interferon-based treatment on hepatitis B virus genotypes

Ref.	Study type	Number of patients (treated/controls)	IFN	Region of origin	HBeAg-positive (%)	Treatment endpoint	Treatment endpoint by HBV genotype (%)	Significance (*P* value)
Wai *et al*[26]	Retrospective, unmatched controls	107 (73/34)	IFN α	China	100.0	HBeAg seroconversion	B: 39 C: 17	0.03
Kao *et al*[27]	Retrospective, unmatched controls	58 (58/0)	IFN α	Taiwan	100.0	Loss of HBeAg and HBV DNA 48 wk post-treatment	B: 41 C: 15	0.045
Zhang *et al*[28]	Retrospective, unmatched controls	35 (35/0)	IFN α	France	0.0	Normalization of ALT, loss of HBV DNA by a branched-DNA assay 6 mo post-treatment	A: 70 non-A: 40	0.001
Erhardt *et al*[29]	Retrospective, unmatched controls	165 (69/72)	IFN α	Germany, Mediterranean, Eastern Europe, Asia, Africa	A: 51.4 B: 5 C: 8.4 D: 31.9 E: 2.5 G: 0.8	HBeAg-positive: normalization of ALT, negative HBV DNA by a hybridization assay, and HBeAg seroconversion 6 mo post-treatment HBeAg-negative: loss of HBV DNA by a hybridization assay and the normalization of ALT 6 mo post-treatment	(overall) A: 49 D: 26 (HBeAg-positive) A: 46 D: 24 (HBeAg-negative) A: 59 D: 29	0.005 0.03 0.05
Flink *et al*[30]	Prospective, multicenter randomized controlled trials	266 (266/0)	Peg- IFN-α-2b +/- lamivudine	The Netherlands	100.0	Loss of HBsAg at the end of follow-up	A: 14 B: 9 C: 3 D: 2	0.006
Erhardt *et al*[32]	Retrospective, cohort	49 (23/26)	IFN α	Germany, Hong Kong, France	E: 47 F: 50 G: 50 H: 60	Normalization of ALT, decrease of HBV DNA < 4000IU/mL 6 mo post-treatment	E: 36 F: 50 G: 20 H: 50	NA

IFN: Interferon; HBeAg: Hepatitis e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; NS: Not significant; NA: Not available.

patients (49% *vs* 26%, $P < 0.005$). The sustained response rate to IFN was 46% *vs* 24% ($P < 0.03$), respectively, in patients with HBeAg positive hepatitis and was 59% *vs* 29% ($P < 0.05$) for HBV genotype A and HBV genotype D, respectively, in patients with HBeAg negative hepatitis.

Large multicenter trials of pegylated IFN-α revealed a significant correlation between the viral genotype and sustained HBeAg loss in patients treated with pegylated-IFN-α-2b. The highest rate of HBeAg clearance at the end of the follow-up was observed in patients infected with genotype A (47%), followed by genotypes B (44%), C (28%), and D (25%). Further analyses of the same study population demonstrated that HBsAg clearance was also closely linked to the viral genotype and was the highest in genotype A (14%), followed by B (9%), C (3%), and D (2%)[30]. A recent meta-analysis has provided compelling support of genotype A as the most treatment-responsive genotype in HBeAg-positive hepatitis B[31].

The antiviral responses of HBV genotypes E-H have not been examined in detail. In a small study from Germany, 23 patients with HBV genotypes E-H received IFN-α[32]. A SVR was defined as the normalization of ALT and decrease in HBV DNA of < 4000 IU/mL 6 mo after treatment. SVR was 35% (8/23) for patients treated with IFN-α. SVR was 36% (5/14) for HBV genotype E, 50% (2/4) for HBV genotype F or H, and 20% (1/5) for HBV genotype G. HBV genotype G/A co-infection was found in 40% of patients with HBV genotype G, whereas HBV genotype G/C co-infection was found in 30%. HBV genotypes E, F, and H appeared to be sensitive to IFN-α. The lower response rates to IFN-α observed in patients with HBV genotype G may be related to the frequent occurrence of double infection. In a study from Argentina, Marciano *et al*[33] reported that although the available data were even more limited, HBV genotype F had a more similar response rate to pegylated IFN-α-2a than HBV genotype A.

The precore mutation ($G_{1896}A$) and dual core promoter mutations ($A_{1762}T$ and $G_{1764}A$) play an important role in the molecular virological factors that contribute to clinical outcome and therapy for chronic hepatitis B. These mutations are closely associated with the seroconversion of HBeAg[34,35]. Although the rate of dual core promoter mutations has been shown to be higher in genotype C and closely related to the active progression of chronic liver disease and increased risk for HCC[12,36-38], studies have reported a correlation between these mutations and a better response to IFN-α therapy[39-41].

Table 2 Summary of the long-term follow-up studies of interferon-α treatment on hepatitis B virus-related liver disease progression

Ref.	Study type	Number of patients (treated/ controls)	Region of origin	HBeAg-positive	Treatment follow-up (yr)	Cirrhosis development (% treated *vs* % controls)	Significance (*P* value)	Cirrhosis complication development (% treated *vs* % controls)	Significance (*P* value)
Lin *et al*[19]	Prospective, randomized, controlled	89 (60/29)	Taiwan	100%	7.4	13.3 *vs* 17.2	NS	9.0 *vs* 14.7	NS
Lin *et al*[42]	Retrospective, matched controls	466 (233/233)	Taiwan	100%	6.8	17.8 *vs* 33.7	0.041	NA	NA
Krogsgaard *et al*[43]	Retrospective, multicenter controlled trials	253 (histologically evaluated)	Europe	100%	4.7	10 *vs* 10	NS	NA	NA
Tangkijvanich *et al*[44]	Retrospective, unmatched controls	139 (69/72)	Thailand	100%	5.0	10.4 *vs* 22.2	NS	NA	NA
Truong *et al*[46]	Retrospective, matched controls	62 (27/35)	Japan	59.6%	6.5	11 *vs* 6	NS	NA	NA
Yuen *et al*[47]	Prospective, matched controls	411 (208/203)	Hong Kong	100%	8.9	NA	NA	4.3 *vs* 1.0	0.062

HBeAg: Hepatitis e antigen; NS: Not significant; NA: Not available.

LONG-TERM OUTCOME ON LIVER DISEASE PROGRESSION

Long-term follow-up studies after a 4- to 6-mo course of IFN therapy in HBeAg-positive patients showed a reduction in the progression of fibrosis, especially in patients with sustained HBeAg seroconversion[17,19,20]. Table 2 summarizes the details of long-term follow-up studies of IFN-α treatment on HBV-related liver disease progression.

Lin *et al*[19] conducted follow-up of 101 HBeAg-positive male Taiwan patients. In 89 patients without any evidence of cirrhosis on admission, cirrhosis developed in 1 (4.2%) of the 24 seroconverters and 7 (19.4%) of the 36 nonseroconverters in the treated group, and 5 (23.8%) of the 21 nonseroconverters in the untreated group, whereas cirrhosis did not develop in any of the 8 seroconverters in the untreated group ($P > 0.05$). No significant differences were observed in the incidence of new cirrhosis (13.3% of treated patients *vs* 17.2% of untreated patients) and in complications of cirrhosis (9.0% of treated patients *vs* 14.7% of untreated patients) between the two groups of patients.

The study expanded their sample population by including patients from other nonrandomized studies in Taiwan[42]. This large study compared 233 IFN-treated HBeAg-positive patients with 233 well-matched untreated patients and reported a reduction in the cumulative incidence of cirrhosis (17.8% *vs* 33.7% in the controls; $P = 0.041$) after a median follow-up of 6.8 (1.1-16.5) years.

Krogsgaard *et al*[43] conducted a follow-up study of 469 HBeAg-positive patients from three multicenter European trials. 253 patients were histologically evaluated. No significant difference was observed in progression to cirrhosis between the treated and untreated groups (10% *vs* 10%).

Tangkijvanich *et al*[44] conducted follow-up of 139 HBeAg-positive patients in a retrospective study in Thailand. The baseline ALT levels of the two groups were unmatched, which were higher in the treated group ($P = 0.001$). Progression to cirrhosis was observed in 4.2% of sustained responders and 14% of nonresponders, whereas 22.2% of controls. The overall incidence of new cirrhosis in sustained responders was significantly lower than that in the control group ($P = 0.04$).

Papatheodoridis *et al*[45] prospectively followed-up 406 non-randomized HBeAg-negative patients in Greece. The baseline ALT levels in the treated patients were significantly higher than those in the untreated patients ($P < 0.001$), and no significant differences were observed in survival or complication-free survival between IFN-α-treated patients and untreated patients ($P = 0.62$ and $P = 0.14$, respectively). Survival and complication-free survival were significantly better in sustained responders than in non-sustained responders ($P = 0.027$ and $P = 0.019$, respectively) or untreated patients ($P = 0.048$ and $P = 0.012$, respectively).

In a follow-up study of 62 Japanese patients conducted by Truong *et al*[46], the baseline ALT levels of the two groups were unmatched, which were significantly higher in the treated group than in the untreated group ($P < 0.05$). No significant difference was observed in HBeAg seroconversion, ALT normalization, and undetectable HBV DNA between the two groups of patients. Progression to cirrhosis was observed in 3 treated patients (11%) and 2 untreated patients (6%).

In a case-controlled study of IFN-α *vs* no treatment in patients recruited from 4 previous randomized controlled trials in Hong Kong, Yuen *et al*[47] conducted followed-up of 208 HBeAg-positive patients treated with IFN-α and compared them to 203 controls. No significant differences were observed in the HBeAg seroconversion rate or undetectable HBV DNA by PCR assay

Table 3 Summary of the long-term follow-up studies of interferon α treatment on the development of hepatitis B virus-related hepatocellular carcinoma

Ref.	Study type	Number of patients (treated/ controls)	Region of origin	HBeAg-positive (%)	Cirrhosis (%)	Treatment follow-up (yr)	HCC development (% treated *vs* % controls)	Significance (*P* value)
Lin *et al*[19]	Prospective, randomized, controlled	101 (67/34)	Taiwan	100	12.5	7.4	1.5 *vs* 11.8	0.043
Lin *et al*[42]	Retrospective, matched controls	466 (233/233)	Taiwan	100	9.4	6.8	2.7 *vs* 12.5	0.011
Ikeda *et al*[49]	Retrospective, unmatched controls	313 (94/219)	Japan	36	100	6.9	17.0 *vs* 30.8	0.012
Krogsgaard *et al*[43]	Retrospective, multicenter controlled trials	308 (210/98)	Europe	100	6.1	4.7	0.96 *vs* 1.0	NS
International IFN-α HCC Study Group[50]	Retrospective, multicenter, matched controls	146 (49/97)	Italy, Argentina	NA	91	6.5	16 *vs* 19	NS
Mazzella *et al*[51]	Prospective, randomized, controlled	64 (33/ 31)	Italy	0	0	6.9	3.0 *vs* 6.4	NS
Tangkijvanich *et al*[44]	Retrospective, unmatched controls	139 (69/72)	Thailand	100	20.1	5.0	4.7 *vs* 12.5	NS
Papatheodoridis *et al*[45]	Retrospective, unmatched controls	404 (209/195)	Greece	100	30.9	6.0	8.1 *vs* 7.7	NS
Truong *et al*[46]	Retrospective, matched controls	62 (27/35)	Japan	59.6	1.6	6.5	3.7 *vs* 0.0	NS
Yuen *et al*[47]	Prospective, matched controls	411 (208/203)	Hong Kong	100	NA	8.9	2.4 *vs* 0.0	NS
			Number of analyzed studies		**RD/RR**		**95%CI**	**Significance (*P* value)**
Cammà *et al*[52]	Meta-analysis	853/652	7 Studies		RD = -6.4% (overall)		-2.8%-(-10%)	< 0.001
					RD = -4.8% (European)		-11.1%-1.5%	NS
Miyake *et al*[53]	Meta-analysis	553/750	8 Studies		RD = -5.0% (overall)		-9.4%-(-0.5%)	0.028
					RD = -8.5% (Asian)		-13.6-(-3.6%)	0.001
Sung *et al*[54]	Meta-analysis	1292/1458	12 Studies		RR = 0.66		0.48-0.89	0.006
Yang *et al*[55]	Meta-analysis	1006/1076	11 Studies		RR = 0.59		0.43-0.81	0.001
Zhang *et al*[56]	Meta-analysis	176/171	2 Studies		RR = 0.23		0.05-1.04	NS
Jin *et al*[57]	Meta-analysis	1291/1048	9 Studies		RR = 0.274		0.059-1.031	NS

IFN: Interferon; HBeAg: Hepatitis e antigen; HCC: Hepatocellular carcinoma; NS: Not significant; NA: Not available; RD: Risk difference; RR: Relative risk.

between the two groups of patients. Complications associated with cirrhosis developed in 9 treated patients (4.3%) and in only 2 untreated patients (1.0%) ($P = 0.062$).

LONG-TERM OUTCOME ON THE DEVELOPMENT OF HCC

Studies on the long-term effect of IFN on the prevention of HCC in patients with HBV have yielded conflicting results. The utility of IFN-α in preventing HBV-related HCC, unlike its effect on hepatitis C virus (HCV)-related HCC, is highly variable among different studies. Most studies are limited by a relatively small sample size, short duration of follow-up, and lack of a control group for comparisons. Table 3 summarizes the details of long-term follow-up studies of IFN-α treatment on the development of HBV-related HCC.

Three studies have reported the beneficial effect of IFN on HBV-related HCC prevention[48]. The first report by Lin *et al*[19] was a follow-up study of 101 HBeAg-positive male patients in Taiwan. When recruited, cirrhosis was present in 10.3% of the treated patients and 14.7% of the untreated patients. HCC developed in 1 of the treated patients (1.5%) and 4 of the untreated patients (11.8%) ($P = 0.043$).

The second report was a large matched control study of HBeAg-positive patients with active chronic hepatitis B conducted by the same group of investigators who subsequently extended their study population in Taiwan[42]. This large study compared 233 IFN-treated HBeAg-positive patients with 233 well-matched untreated patients and showed a reduction in the incidence of HCC (2.7% *vs* 12.5% in the controls, $P = 0.011$) after a median follow-up of 6.8 (1.1-16.5) years. When recruited, 8.1% of the treated patients and 10.7% of the untreated patients had cirrhosis. Significant reduction in the incidence of HCC was only observed in patients with cirrhosis of the liver on recruitment (19.7% for treated patients and 58.9% for untreated patients; $P < 0.01$). The reduction in

the incidence of HCC was not observed in the patients without cirrhosis (2.1% for treated patients and 2.3% for untreated patients, not significant). Both of the studies did not show the significant relationship between HBV DNA suppression and HCC development with IFN-α treatment.

The third study to demonstrate that IFN-α was useful in reducing the incidence of HCC was conducted in patients with cirrhosis only in Japan[49]. Cumulative occurrence rates of HCC were 4.5%, 7.0%, and 17% at the end of 3, 5, and 10 years, respectively, for IFN-treated patients, 13.3%, 19.6%, and 30.8% for controls. The IFN-α treatment significantly decreased the rate of HCC development ($P = 0.0124$). The Cox proportional hazard model revealed that IFN-α treatment was an independent contributing factor that lowered the rate of carcinogenesis (OR = 0.39, $P = 0.031$) even after a correction by significant covariates in multivariate analysis. In this study, the reduction in the incidence of HCC by the IFN-α treatment was significantly higher in patients with a viral load higher than 10^6 copies/mL.

Seven studies did not show any beneficial effect of IFN on the progression of HCC[48]. Krogsgaard *et al*[43] conducted follow-up study of 469 HBeAg-positive patients from three multicenter European trials. HCC developed in three patients (1%) during the follow-up period: 2 were treated with IFN and 1 was untreated. The distribution of clinical events (progression to cirrhosis, HCC and liver-related deaths) was unrelated to response to IFN-α treatment.

The International Interferon-α Hepatocellular Carcinoma Study Group retrospectively recruited 913 patients positive for the anti-HCV antibody and/or HBsAg from 21 centers in Italy and Argentina[50]. In 146 of the HBV-infected patients, eight (16%) of 49 treated patients and 18 (19%) of 97 untreated patients developed HCC (RR = 0.98, 95%CI: 0.33-2.92). The risk reduction by IFN-α treatment was apparently greater for patients with chronic hepatitis C and no evidence of HBV infection.

Mazzella *et al*[51] conducted follow-up study of 33 HBeAg-positive Italian patients treated with IFN-α and compared them to 31 controls. IFN-α treatment accelerated the loss of HBeAg (90.9% in treated *vs* 61.3% in control patients, $P < 0.007$) and HBsAg (36.4% *vs* 9.8%, $P < 0.017$), whereas 1 (3.0%) treated patient and 2 (6.4%) controls developed HCC. IFN-α treatment did not have beneficial effect on the loss of HBV DNA (78.9% *vs* 58.1%, $P = 0.106$).

Tangkijvanich *et al*[44] conducted follow-up study of 139 HBeAg-positive patients in Thailand. HCC appeared in 11 cirrhotic patients: 9 (12.5%) in the control group and 2 (4.7%) of the nonresponders ($P > 0.05$). None of the sustained responders ($n = 24$) developed HCC. Investigators reported that IFN-α treatment might prevent the progression of cirrhosis and development of HCC in sustained responders. However, the lack of significant *P* values renders the conclusions insufficient.

Papatheodoridis *et al*[45] conducted prospective follow-up study of 404 non-randomized HBeAg-negative patients in Greece. The baseline ALT levels in the treated patients were significantly higher than those in the untreated patients ($P < 0.001$), and HCC appeared in 17 (8.1%) treated (16 in nonresponders) and 15 (7.75%) untreated patients. IFN-α improved long-term outcome (liver decompensation and/or HCC) in patients with sustained biochemical remission, even in the presence of cirrhosis and old age.

In a follow-up study of 62 Japanese patients, Truong *et al*[46] found that although one (3.7%) treated patient developed HCC, none of the controls developed HCC. The baseline ALT levels in the treated patients were significantly higher than those in the untreated patients ($P < 0.05$). Most patients continued to have detectable HBV DNA by PCR assay after HBeAg seroconversion.

In a case-control study of IFN-α *vs* no treatment in patients recruited from 4 previous randomized controlled trials in Hong Kong, Yuen *et al*[47] followed-up 208 HBeAg-positive patients treated with IFN-α and compared them to 203 controls. The development of HCC was observed in five (2.4%) treated patients and none of the untreated patients.

Six meta-analyses[52-57] on the effect of IFN-α treatment on the development of HBV-related HCC have been reported. Studies by Cammà *et al*[52] and Miyake *et al*[53] calculated the risk difference (RD), whereas the other four studies[54-57] calculated the relative risk (RR). Cammà *et al*[52] reported a different incidence of HCC between treated and untreated HBV-related Child A cirrhotic patients (overall RD = -6.4%; 95%CI: -2.8%-(-10%), $P < 0.001$); however, no preventive effect of HCC was shown for HBV when data from European studies were evaluated (overall RD = -4.8%: 95%CI -11.1 - 1.5%, not significant). Therefore, it was concluded that IFN did not appear to affect the rate of HCC in HBV-related cirrhosis.

Miyake *et al*[53] demonstrated the preventive effect of IFN treatment in an Asian population (RD = -8.5%; 95%CI: -13.6-(-3.6), $P = 0.0012$), but not in a European population. These findings indicate that IFN treatment suppressed the development of HCC in patients with chronic hepatitis B virus infection, especially in HBeAg-positive Asians.

Sung *et al*[54] showed that the risk of HCC after IFN treatment was reduced by 34% (RR = 0.66, 95%CI: 0.48-0.89) and that this effect was significantly strongly in patients with early cirrhosis than in those without cirrhosis.

Yang *et al*[55] reported a difference in the incidence of liver cirrhosis and HCC between treated and untreated patients (RR = 0.65, 95%CI: 0.47-0.91, RR = 0.59, 95%CI: 0.43-0.81, respectively). These findings indicated that IFN prevents or delays the development of liver cirrhosis and HCC in patients with chronic hepatitis B.

Zhang *et al*[56] demonstrated that IFN did not significantly affect the overall rate of HCC in HBV-infected patients; however, IFN therapy slightly ameliorated the rate of HCC (RR = 0.23, 95%CI: 0.05-1.04, $P = 0.056$).

Jin *et al*[57] reported that IFN was associated with significant preventive effects on HCC according to the DerSimonian-Laird method (RR = 0.470, 95%CI: 0.260-0.850) and Bayesian methodology, with an adjustment for the underlying risk [RR = 0.249, 95% Bayesian credible intervals (BCI): 0.049-0.961] but not according to a Bayesian meta-analysis (RR = 0.274, 95%BCI: 0.059-1.031). These findings indicate that additional evidence is needed to support the role of IFN in delaying the progression of chronic hepatitis B.

Long-term follow-up studies of IFN-α treatment on the development and progression of HCC show inconsistent results. The preventive effect on HCC of IFN-α treatment has mainly been observed in sustained responders to the treatment who already have cirrhosis. However, IFN-α may lead to severe liver decompensation when used in patients with cirrhosis, and only less than 35% of treated patients are sustained responders. Almost all current guidelines suggest that patients with HBV-related cirrhosis should receive nucleoside/nucleotide analog therapy. These inconsistent findings may be attributed to the inevitable limitations of comparisons across studies, including differences in the baseline characteristics of the study and the moderate suppression of HBV replication by IFN-α relative to nucleoside/nucleotide analogs.

CONCLUSION

The ability of IFN-α treatment to prevent new cirrhosis, complications associated with cirrhosis, and development of HCC over the long-term is controversial. These inconsistent findings may be due to the inevitable limitations of comparisons across studies, including differences in the baseline characteristics of the study and the moderate suppression of HBV replication by IFN-α relative to nucleoside/nucleotide analogs.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Update on hepatitis B virus infection

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Abstract

Chronic infection with hepatitis B virus (HBV) leads to the development of hepatocellular carcinoma and/or chronic liver failure. Despite extensive research, the immunopathogenesis is not completely understood. Viral persistence and clinical outcomes following HBV infection depend on viral factors and host factors; including genetic factors that determine a host's immune mechanisms. The primary goal of chronic hepatitis B (CHB) treatment is to eradicate HBV or to at least maintain suppression of HBV replication. Despite recent advances in anti-viral agents for chronic HBV infection, complete eradication of the virus has been difficult to achieve. Agents for the treatment of CHB are divided mainly into two groups: immunomodulating agents and antiviral nucleos(t)ide analogues (NAs). Although NAs are safe, effective and easily administered orally, their long-term use poses the risk of drug resistance. Currently, international evidence-based guidelines have been developed to support physicians in managing CHB patients. However, treatment of patients with drug resistance is still challenging, as only a few classes of anti-HBV drugs are available and cross-resistance between drugs can occur. In addition, as the currently available genotypic test for detection of drug resistance still has limitations in identifying the different substitutions present in the same viral genome, the development of a new virologic test to overcome this limitation is necessary. Among the predictive factors associated with response to pegylated interferon (PEG-IFN) therapy, hepatitis B surface antigen quantification is considered to be a surrogate marker for monitoring response to PEG-IFN. Current practice guidelines stress the importance of profound and durable HBV viral suppression in the treatment of CHB patients. To this end, it is essential to choose a potent antiviral drug with a low risk of resistance for initial treatment of CHB to achieve sustained virological response. This review highlights recent advances in the understanding of the immunopathogenesis of HBV and currently available and developing treatment strategies against HBV infection.

Key words: Hepatitis B virus; Chronic hepatitis B; Nucleos(t)ide analogue; Pegylated interferon; Antiviral therapy

Core tip: The primary goal of chronic hepatitis B (CHB) treatment is to eradicate hepatitis B virus (HBV) or to at least maintain suppression of HBV replication. To this end, it is essential to better understand the immunopathogenesis of CHB, although it has yet to be further elucidated. More practically, it is of great importance to choose a potent antiviral drug with a low risk of resistance for initial treatment of CHB to achieve sustained virological response. This review highlights recent advances in the understanding of the immunopathogenesis of HBV and currently available and developing treatment strategies for HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV), a member of the family Hepadnaviridae, has a 3.2 kb-partially double-stranded circular DNA genome comprised of 4 open reading frames (ORF) that encode Core proteins (Core and preCore), surface antigen proteins (PreS1, PreS2, and S), reverse transcriptase (Pol protein) and X protein. HBV is a non-cytopathic virus that induces liver damage *via* immunopathogenesis[1].

Despite the introduction of prophylactic vaccines against HBV in the early 1980s, it is estimated that there are still more than 350 million chronic HBV carriers worldwide[2], a high percentage of whom will eventually develop liver cirrhosis or hepatocellular carcinoma (HCC). The natural history of chronic HBV infection is generally divided into four phases: (1) immune tolerant phase; (2) immune clearance phase; (3) low replicative or inactive carrier stage; and (4) reactivation phase[1,3]. Recent studies have shown that progression to liver cirrhosis and HCC in patients with chronic HBV infection is significantly associated with circulating HBV-DNA levels[4,5]. Thus, antiviral therapy against HBV is critical to prevent the progression to cirrhosis or development of HCC. The primary goal of CHB treatment is to eradicate HBV or to at least maintain a suppressed state of HBV replication. However, antiviral therapy is not recommended for patients in the immune tolerant phase, which is characterized by high HBV-DNA levels with positive hepatitis B e antigen (HBeAg), but normal alanine aminotransferase (ALT) level and minimal necroinflammation. In general, antiviral therapy is considered for patients in the immune clearance phase and the reactivation phase of chronic HBV infection.

Since the introduction of interferon (IFN)-α as the first approved agent for HBV infection in the early 90's, remarkable advances have been made in the treatment of CHB. Agents for the treatment of CHB are divided mainly into two groups according to their mechanism of action: (1) agents with immunomodulatory and antiviral effects, such as IFN or peglyated IFN (PEG-IFN); and (2) oral nucleos(t)ide analogues (NAs) such as nucleoside analogues including lamivudine (LAM), telbivudine (LdT), clevudine and entecavir (ETV) and nucleotide analogues including adefovir dipivoxil (ADV) and tenofovir dipivoxil fumarate (TDF). These NAs can be divided into subclasses based on their structural similarities: L-nucleoside analogues (LAM, LdT and Clevudine); alkyl phosphonates (ADV and TDF); and D-cyclopentane (ETV).

The main difference between immunomodulatory agents and NAs is that PEG-IFN has the advantage of a finite duration of use, whereas the use of NA inhibitors is indefinite. The major drawback of PEG-IFN is its high frequency of adverse events. Long-term use of NAs, on the other hand, poses the risk of drug resistance. They are, however, safe, effective and easily administered orally. The number of patients having a virological response after a cycle of IFN therapy is lower compared with patients reaching the suppression of viral replication with new NAs. However, IFN therapy has higher rates of HBeAg seroconversion and hepatitis B surface antigen (HBsAg) loss than NAs. Treatment strategies with PEG-IFN or a NA are intended to achieve a sustained off-treatment virological response. A 48-wk course of PEG-IFN is mainly recommended for HBeAg-positive CHB patients with the best chance of HBeAg seroconversion. It can also be administered in HBeAg-negative CHB patients. Unlike NAs, PEG-IFN potentially offers a chance of sustained off-treatment response after a finite duration of therapy in HBeAg-negative patients. For HBeAg-positive CHB patients, NA therapy can be stopped after additional 12 mo following HBeAg seroconversion, whereas long-term use of NA is needed due to a high rate of off-therapy relapse in HBeAg-negative patients, in whom the ideal end point is HBsAg loss.

LAM, the first approved oral NA for hepatitis B treatment, had been widely used and is a safe and effective drug, however, it has been excluded from most recent international guidelines as a first-line antiviral agent against HBV due to the emergence of drug-induced mutant HBV. The frequency of the development of genotypic resistance was reported to be up to 60%-70% after 5 years of LAM therapy[6,7]. In order to achieve more curative antiviral effects, new therapeutic approaches, such as combination therapies using an antiviral and an immunomodulating agent or multiple antiviral agents used together, have been tried to address this problem, but evidence demonstrating that combination therapy is superior to monotherapy is still lacking[8,9].

The development of a new antiviral agent or optimized therapeutic approaches that can eradicate the virus and not only achieve control of viral replication, should be the focus of future work in the management of CHB. To this end, it is essential to understand the immunopathogenesis of HBV and the mechanisms of viral persistence and drug-induced resistance. The following summarizes recent advances in the understanding of the immunopathogenesis of HBV and currently available and developing optimized antiviral strategies therapies for HBV infection.

IMMUNOPATHOGENESIS

HBV infection in humans results in various clinical outcomes depending on the age of the patient at the time of infection: 90% of individuals infected *via* vertical or perinatal transmission develop chronically evolving hepatitis, while 90% of individuals infected *via* horizontal transmission during adulthood recover[1]. Viral persistence and clinical outcomes following HBV infection depend on viral factors and host factors; including genetic factors that determine a host's immune mechanisms[10].

The innate immune response controls initial infection during the early phases of viral infection. Various components, such as natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, cytokines, chemokines and toll-like receptor (TLR) contribute to this nonspecific innate immune response[11]. Type 1 IFNs produced by infected cells also play an important role in the inhibition

of viral replication early on, but do not provide long-term protective immunity against viruses[11]. Dysfunction of the innate immune response in the immune tolerant phase may be explained by previous studies which showed that HBV suppress TLR-mediated innate immune responses in murine non-parenchymal liver cells[12] and HBeAg reduces TLR2 expression on hepatocytes, Kupffer cells (KCs) and peripheral monocytes in patients with CHB[13]. On the other hand, in the immune clearance phase, NK cell activation induces tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated death of hepatocytes in CHB, leading to liver injury[14]. Interestingly, a recent study by Zhang *et al*[15] demonstrated that peripheral CD16+ monocytes in chronic HBV infection are selectively recruited into the liver, where they are highly activated and produce pro-inflammatory cytokines and induce Th17 cells to initiate destructive immune responses.

Major histocompatibility complex (MHC) class-I restricted CD8+ T-cells and MHC class-Ⅱ restricted CD4+ T-cells have been shown to play key roles in the adaptive cellular immune response against HBV. Studies have shown that individuals who demonstrated spontaneous recovery from HBV infection mount a vigorous, multi-specific T-cell response against HBV antigens. Chronic HBV carriers mount a defective T-cell response, which is characteristically weak and mono-specific, and go on to develop chronic liver diseases such as liver cirrhosis and HCC[16,17]. CD8+ T cells cause the lysis of HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms[18]. The cytolytic mechanism was demonstrated in HBV-transgenic mouse models that express HBV antigens in the liver[19,20]. These transgenic mice are tolerant of self-HBV antigens, and notable liver damage does not develop. However, when cytotoxic T-cells from one of these transgenic mice are introduced into syngenic mice, significant acute liver injury that resembles acute hepatitis B in humans occurs[21]. The lysis of HBV-infected hepatocytes occurs *via* perforin or Fas systems which lead to viral clearance. Non-cytolytic mechanisms are mediated by some specific cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α which selectively degrade replicating genomes of HBV without killing infected cells[21,22]. Since HBV is a non-cytopathic virus, it must be able to either evade or overwhelm antiviral immune responses. Most neonates who are infected *via* vertical transmission from their mothers develop chronically evolving hepatitis through neonatal tolerance. Explanations for this neonatal tolerance involve the immature immune system of neonates and the immunomodulating effects of excessively secreted HBeAg in peripheral blood. This tolerating effect of HBeAg has been well demonstrated in HBV transgenic mice[23]. In addition, studies have shown that cytotoxic T cell responses in patients with chronic HBV infection occur less vigorously than those with acute hepatitis B, even if they have a high viral load in the peripheral blood[16]. This low cytotoxic T cell response is responsible for the induction of peripheral tolerance or the exhaustion of the T cell response due to a high viral load[24]. Moreover, antigen persistence is a major factor driving the functional alteration of HBV-specific T cells, and thus antigen removal to allow T cell resting can be an essential requirement for the functional reconstitution of antiviral T cell responses[25]. The hyporesponsiveness of HBV-specific CD8+ T cells results in decreased cytotoxicity, proliferation, and production of anti-viral cytokines; including TNF-α and interleukin-2 (IL-2), which are responsible for eradicating the virus during chronic infection[26,27]. In addition to HBV-specific T cells, NK cells, NKT and macrophages can inhibit HBV replication by producing IL-12 and nitric oxide[28]. The other factors influencing HBV-specific tolerance include the immunoregulatory effects of CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) and the dysfunction of dendritic cells[29]. Tregs suppress effector T cells, which impair the immune response. Thus, a change in number or function could cause a decrease in anti-viral immunity in chronic HBV carriers. Studies on the frequency of circulating Tregs in chronic HBV carriers have yielded conflicting results-both increased[30] and decreased[31] frequencies have been observed. However, depletion of Tregs increased the function of HBV specific T-cells, even though such modulation was not necessarily HBV-specific[30,31]. Hence, the role of Tregs in the immunopathogenesis of HBV should be further clarified.

Although the immunomodulatory effect of chronic HBV infection on dendritic cell function is poorly understood, recent studies suggest that dysfunction of dendritic cells may contribute to inhibition of the antiviral immune response, leading to viral persistence and progressive liver disease in chronic HBV carriers[32]. In addition, recent studies have shown that the programmed death-1 (PD-1) inhibitory receptor is involved in the impairment of virus-specific CD8 T-cell function in chronic viral infections in mice and humans[33,34]. Little data is available on the association between PD-1 expression and HBV-specific CD8+ T cells, but a recent study has demonstrated that the increase in HBV-specific CD8+ T cell proliferation correlates with the downregulation of PD-1[35]. Based on these experiments, it can be surmised that blocking the activation of PD-1 can restore exhausted virus-specific CD8+ T cells and thus lead to a promising strategy for treating chronic HBV infection. Cytotoxic T cells play a crucial role in controlling viral replication, but selection for viral escape mutations in targeted T-cell epitopes may limit their effectiveness. However, viral escape mutants in HBV have rarely been reported, even in cases of CHB[36], with the exception of a few reports which demonstrated that viral mutations abrogate, energize or antagonize antigen recognition by virus-specific T cells[37,38]. Other possible mechanisms leading to HBV persistence include the infection of immunologically privileged sites, viral inhibition of antigen presentation and downregulation of viral gene expression[24]. The immune responses against HBV and its regulation are summarized in Figure 1.

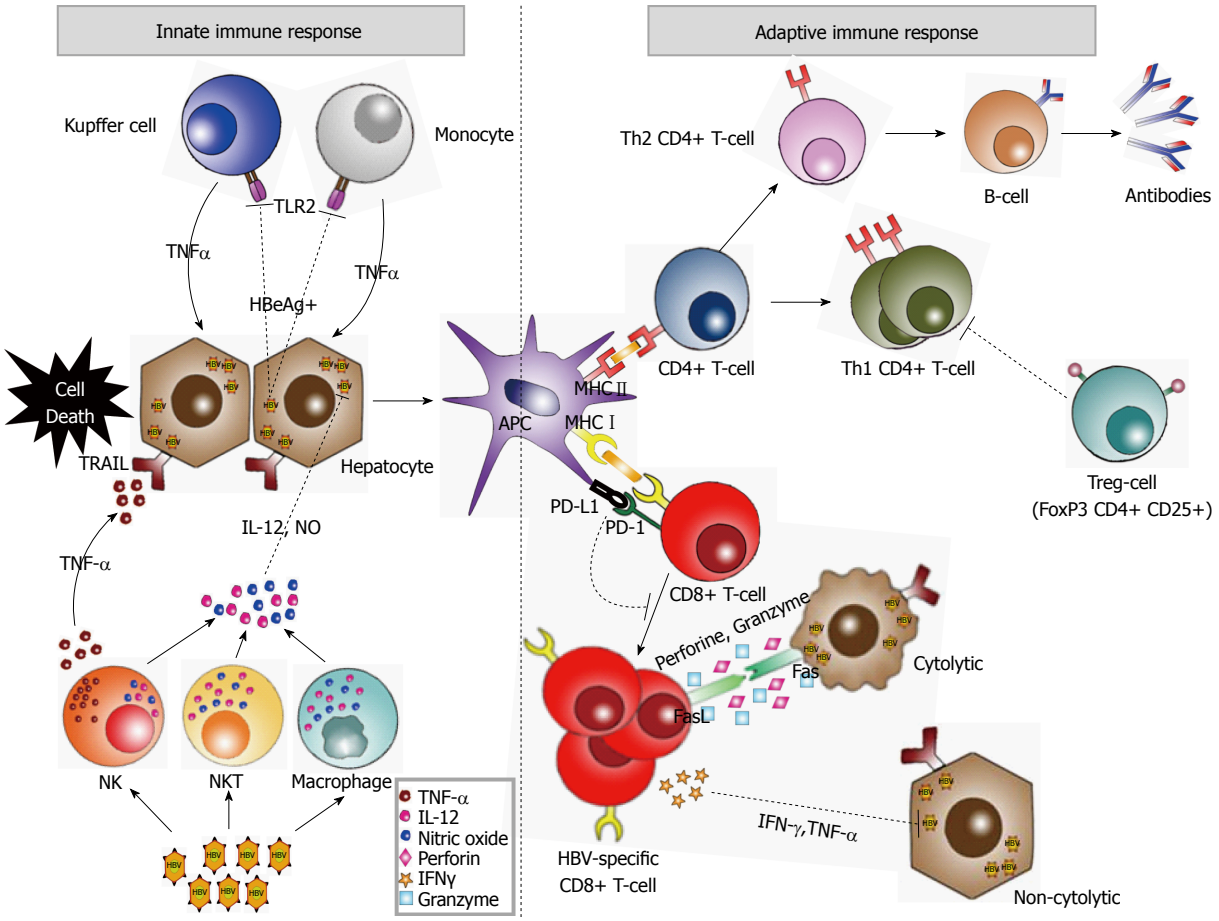

Figure 1 Summary of the innate immune response and cellular immune response against hepatitis B virus. Natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, cytokines, chemokines and toll-like receptor (TLR) contribute to the innate immune response against hepatitis B virus (HBV). Also, NK cells, NKT and macrophages can inhibit HBV replication by producing interleukin (IL)-12 and nitric oxide. Dysfunction of the innate immune response in the immune tolerant phase may be explained by this process. However, HBeAg reduces TLR2 expression on hepatocytes, Kupffer cells and peripheral monocytes. Moreover, NK cell activation induces TRAIL-mediated death of hepatocytes in CHB, leading to liver injury. Chronic HBV carriers mount a defective T-cell response. CD8+ T cells cause the lysis of HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms. Non-cytolytic mechanisms are mediated by some specific cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α which selectively degrade replicating genomes of HBV without killing infected cells. In CHB, Tregs suppress effector T cells, which impair the cellular immune response. The programmed death-1 (PD-1) inhibitory receptor is involved in the impairment of virus-specific CD8 T-cell function.

UPDATE ON THE TREATMENT OF CHRONIC HEPATITIS B

The ultimate goal of antiviral therapy for CHB is to prevent progression to cirrhosis or HCC *via* eradication of HBV or persistent viral suppression. The ideal end point of antiviral therapy is the achievement of either HBsAg seroconversion or HBsAg loss. Therefore, antiviral treatment is recommended for patients with chronic HBV infection who are at risk of developing liver failure or for those with advanced liver diseases[39,40]. Regional clinical practice guidelines for the treatment of patients with chronic HBV infection have recently been developed and updated: American Association for the Study of Liver Disease (AASLD) in 2009[40], The Asian Pacific Association for the Study of the Liver (APASL) in 2012[39] and European Association for the Study of the Liver (EASL) in 2012[41]. In deciding upon an appropriate treatment plan for patients with chronic HBV infection, careful consideration must be given to the degree of liver disease, laboratory data, including biochemical and viral levels, histological findings and patient compliance. Although recommendations for the management of chronic HBV infection are similar between regional guidelines, there are some differences regarding the levels of HBV-DNA and ALT, examination of liver biopsy prior to treatment for CHB and liver cirrhosis (Table 1). Among currently available antiviral agents including the standard IFN, PEG-IFN, LAM, ADV, LdT, ETV, TDF and clevudine, PEG-IFN-based therapy, ETV and TDF are recommended for naïve patients with CHB according to the regional guidelines. For patients with liver cirrhosis, however, AASLD guidelines recommend ETV, TDF or the combination of LAM (or LdT) and ADV, while EASL and APASL guidelines recommend ETV and TDF for decompensated liver cirrhosis, and to consider IFN-based therapy for compensated liver cirrhosis. On the other hand, antiviral therapy is not recommended for patients in the immune tolerant phase or the inactive carrier state as disease progression is rare in these phases, and treatment would induce an undue risk of developing drug resistance in such patients.

Table 1 Indications for treatment and recommended strategy for chronic hepatitis B

		AASLD 2009	EASL 2012	APASL 2012
HBeAg positive hepatitis	Indications for treatment	HBV DNA > 20000 IU/mL and ALT > 2 × ULN	HBV DNA > 2000 IU/mL and ALT > ULN	HBV DNA > 20000 IU/mL and ALT > 2 × ULN
	Biopsy recommended[1]	HBV DNA >20000 IU/mL and ALT 1-2 × UNL especially if age > 40 yr or family history of HCC	HBV DNA > 2000 IU/mL and Age > 30 yr or family history of HCC	HBV DNA > 20000 IU/mL or high normal or minimally raised ALT and age > 40 yr
	Preferred drugs for naïve patients	PEG-IFN, ETV, TDF	PEG-IFN, ETV, TDF	IFN-based therapy, ETV, TDF
HBeAg negative hepatitis	Indications for treatment	HBV DNA >20000 IU/mL[2] and ALT > 2 × ULN	HBV DNA > 2000 IU/mL and ALT > ULN	HBV DNA > 2000 IU/mL and ALT > 2 × ULN
	Biopsy recommended[1]	HBV DNA >2000 IU/mL and ALT 1- > 2 × UNL	Unmentioned	HBV DNA > 2000 IU/mL or high normal or minimally raised ALT and age > 40 yr
	Preferred drugs for naïve patients	PEG-IFN, ETV, TDF	PEG-IFN, ETV, TDF	IFN-based therapy, ETV, TDF
Liver cirrhosis	Indication for treatment	Compensated: HBV DNA >2000 IU/mL; consider treating HBV < 2000 IU/mL if ALT > UNL Decompensated: any detectable HBV DNA	Any detectable HBV DNA	Compensated: HBV DNA > 2000 IU/mL Decompensated: any detectable HBV DNA
	Preferred drugs for naïve patients	Combination of LAM (or LDT) and ADV, ETV, TDF	Compensated: PEG-IFN, ETV, TDF Decompensated: ETV, TDF	ETV, TDF (consider also IFN-based therapy if compensated LC and ALT < 5 × UNL)

[1]Consider treatment if moderate to severe inflammation or significant fibrosis is shown on liver biopsy; noninvasive methods may be useful in EASL and APASL guidelines; [2]Treatment may be considered in patients with HBV DNA 2000-20000 IU/mL, particularly if they are older or have cirrhosis. AASLD: The American Association for the Study of the Liver Diseases; EASL: European Association for the Study of the Liver; APASL: The Asian Pacific Association for the Study of the Liver; ULN: Upper limit of normal; PEG-IFN: Pegylated interferon; ETV: Entecavir; TDF: Tenofovir; IFN: Interferon; LAM: Lamivudine; LDT: Telbivudine; ADV: Adefovir.

Pegylated interferon

The most important advantage of PEG-IFN use in clinical practice is finite duration of treatment. In addition, the antiviral response is more durable with higher HBeAg seroconversion and HBsAg seroconversion after treatment compared with NAs. However, frequent adverse events, low degree of compliance due to injection-related problems[41] such as flu-like symptoms, neutropenia, thrombocytopenia, decreased appetite, abdominal discomfort, rash, pruritus, alopecia, thyroiditis, injection-site reaction and depression, and high costs are considered to be some of its drawbacks. Although PEG-IFN does not result in marked DNA suppression during treatment, it has shown to induce delayed post-treatment anti-viral benefits. In two randomized clinical trials (RCT) investigating the efficacy and safety of PEG-IFN-α-2a in HBeAg positive CHB patients, the HBeAg seroconversion rate at the end-of-treatment was approximately 30% of cases. This virological response increased during the period of follow-up, reflecting post-treatment delayed immune responses[42,43]. In addition, 83% of patients who achieved seroconversion during treatment or early post-treatment had a sustained response at 12 mo post-treatment[43]. Decreasing the viral load by NA treatment prior to an immune modulator may be a rational strategy to achieve a more effective viral response given that simultaneous combination therapies have not shown a superior viral response. However, in both of the above-mentioned RCTs, simultaneous combination therapy with PEG-IFN-α-2a plus LAM was not superior to monotherapy with PEG-IFN-α-2a. Previous clinical studies on the antiviral efficacy of LAM followed by PEG-IFN therapy compared with placebo followed by PEG-IFN in HBeAg-positive CHB patients demonstrated that sequential therapy of LAM and PEG-IFN improved sustained virological response as compared with the control group[44]. However, in another sequential therapy regimen using ETV and PEG-IFN, there was no significant difference in HBeAg and HBsAg seroconversion between ETV plus PEG-IFN sequential therapy and sustained ETV monotherapy in HBeAg-positive CHB patients[45]. This hypothesis requires testing in large randomized clinical trials.

Factors that predict response to PEG-IFN therapy are an area of extensive investigation. These factors include serum HBV-DNA, ALT and HBsAg levels, HBV genotype and IL28 polymorphisms[40,41,43,46]. ALT flares (> 2-5 times the upper limit of normal) during IFN treatment reflecting enhanced host immune response[47] as well as low HBV-DNA are widely accepted as predictors for viral response to PEG-IFN therapy[40,48]. Studies have failed to find an association between HBV genotype and response after PEG-IFN therapy such as the phase Ⅲ study conducted by Piratvisuth *et al*[43] and the NEPTUNE study by Liaw *et al*[49] which found no significant difference in HBeAg seroconversion between HBV genotype B and C groups 24 wk post-PEG-IFN treatment. Baseline HBsAg levels were associated with significantly different responses to PEG-IFN therapy in both studies. In addition, a recent study of HBeAg-positive CHB pa-

tients treated with PEG-IFN showed that HBsAg levels can be confidently used to guide therapy decisions and that discontinuation of PEG-IFN treatment is indicated in all patients with HBsAg levels > 20000 IU/mL after 24 wk of PEG-IFN therapy[46].

An IL28B polymorphism is associated with favorable viral response events such as HBeAg and HBsAg loss or seroconversion in HBeAg-positive CHB patients[46,50]. Furthermore, it was demonstrated that the HBsAg seroconversion rate in HBeAg-negative CHB patients was significantly higher in the IL28B CC group than in the non-CC group after a median of 11 years follow-up[51]. In contrast, no significant difference in HBeAg seroconversion rate was observed between the TT group and non-TT group of the IL28B in another study[52].

The levels of serum HBeAg may be considered as a predictive factor for response to PEG-IFN therapy. A recent study by Fried *et al*[53] showed that levels of HBeAg consistently decreased during treatment in patients who achieved HBeAg seroconversion and remained at their lowest level during the 24 wk of post-treatment follow-up. Taken together, lower HBV-DNA levels, higher ALT levels and lower HBsAg levels are considered strong candidate predictive markers of favorable response to PEG-IFN therapy. However, HBV genotype and IL28B polymorphisms still have limited application as useful predictive biomarkers of response to PEG-IFN therapy. Further RCTs on this issue are needed.

Nucleos(t)ide analogues

LAM: Although LAM is the first approved oral antiviral agent against HBV, the emergence of drug-resistant tyrosine-methionine-aspartate-aspartate (YMDD) mutants has been a major hindrance to long-term LAM treatment. The frequency of genotypic resistance was reported to be as high as 60%-70% after 5 years of treatment[6,7]. Therefore, it is no longer recommended as the first-line treatment for CHB patients in the current international guidelines, but it may be recommended in combination therapies with ADV or TDF for liver cirrhosis[40].

ADV: ADV is the acyclic analogue of dAMP with therapeutic efficacy against both the wild-type and YMDD mutant HBV. Two previous major phase Ⅲ clinical studies showed that ADV demonstrated significant virological and biochemical responses, and histological improvement in HBeAg-positive and -negative naïve CHB patients[54,55]. ADV has also demonstrated virological improvement in patients with LAM-resistant HBV, regardless if used alone or in combination with ongoing LAM therapy[56,57]. Although ADV has been widely accepted as advantageous over LAM in terms of the development of genotypic resistance, it is not recommended as the first-line drug for HBeAg-positive and -negative CHB patients in the current international guidelines as it is less effective than other NAs such as ETV and TDF in antiviral activity[58], and possesses a significant risk of nephrotoxicity[59].

Entecavir: ETV is a deoxyguanosine nucleoside analogue which has highly potent antiviral activity and a high genetic barrier to resistance against HBV. In long-term follow-up studies of ETV for the treatment of chronic HBV infection for up to 5 years, it was very safe and well tolerated, and showed high rates of HBV-DNA suppression, ALT normalization and serological responses in both HBeAg-positive and -negative CHB patients[60,61]. Also, long-term ETV treatment showed the reversal of fibrosis or cirrhosis and continued histological improvement in patients with CHB[61]. Specifically, a post-hoc subgroup analysis in HBeAg-positive and -negative Asian CHB patients treated with ETV for up to 5 years also showed histological improvement with high rates of virological, biochemical and serological responses[62]. These results could provide evidence that ETV is beneficial in Asian CHB patients who have poor prognosis due to perinatal infection with genotype B and C in most cases. Moreover, the emergence of ETV resistance was very low, with a cumulative incidence of ETV resistance rates at year 1, 2, 3, 4, and 5 in treatment-naïve patients of 0.2%, 0.5%, 1.2%, 1.2% and 1.2%, respectively[63]. Based on the results from clinical studies, current regional guidelines have recommended ETV as a first-line therapy for the treatment of compensated and decompensated patients with chronic HBV infection.

LdT: LdT is the L-nucleoside analogue of L-deoxythymidine and displays potent antiviral activity comparable to that of ETV. HBV-DNA was undetectable (< 300 copies/mL) in 60% of HBeAg-positive CHB patients and 88% of HBeAg-negative CHB patients treated with LdT[64]. In addition, ALT normalization was found in 77% and 74%, respectively[65]. HBeAg seroconversion in HBeAg-positive CHB patients was observed in 23% and 30% at year 2 in two studies[64,65]. LdT provides higher rates of virological responses than LAM, but has also been associated with high rates of resistance due to a low genetic barrier. The rates of resistance to LdT at 1 year of treatment were 5% and 2% in HBeAg-positive and -negative CHB patients, and 25% and 11% at year 2, respectively[64,65]. Although LdT was well tolerated and safe, an asymptomatic elevation of creatinine kinase and symptomatic myopathy were occasionally reported. Taken together, LdT is not recommended as a first-line therapy for CHB patients in current international guidelines, but it is recommended as a combination therapy with ADV or TDF for liver cirrhosis[40].

Clevudine: Clevudine is a pyrimidine analogue with potent and sustained antiviral activity against HBV. Previous clinical studies conducted in Korea have demonstrated that clevudine showed potent antiviral activity during therapy and induced a sustained post-treatment antiviral effect for 6 mo after a 12- and 24-wk treatment period[66,67]. Moreover, a double-blind, randomized, clinical study comparing the efficacy and safety of clevudine *vs* LAM for 48 wk in treatment-naïve HBeAg-positive CHB

patients revealed that serum HBV-DNA level was below 300 copies/mL in 73% of patients and that HBeAg seroconversion occurred in 18% of patients at week 48[68]. Although potent viral suppression and sustained antiviral activity after cessation of clevudine were observed, its efficacy has been hampered by the emergence of drug-resistant viral variants. A single mutation at rtM204I confers resistance to clevudine and the incidence of clevudine resistance at 48 wk and 2 years was 1.3%-7% and 24.4%, respectively[69,70]. Furthermore, high rates of symptomatic myopathy due to mitochondrial damage in the muscle have been reported with clevudine[71,72]. For these reasons, clevudine is not recommended as first-line therapy for CHB patients.

TDF: TDF is an acyclic nucleotide inhibitor and has been proved to be a highly powerful antiviral agent against HBV without resistance to TDF during the treatment period[58,73]. In a three-year follow-up study, levels of HBV-DNA below 400 copies/mL at week 144 was observed in 72% of HBeAg-positive and in 87% of HBeAg-negative patients treated with TDF monotherapy[74]. In addition, ALT normalization was found in 74% and 81%, respectively. In HBeAg-positive patients, rates of HBeAg and HBsAg loss were 34% and 8%, respectively. Furthermore, no genotypic resistance developed during the follow-up period. Interestingly, a recent long-term follow-up study over 5 years demonstrated that 87% of patients showed histological improvement and 51% had regression of fibrosis at week 240[75]. In addition, 74% of 96 patients with cirrhosis at baseline had no cirrhosis at year 5. Moreover, no genotypic resistance to TDF was detected. These results provided strong evidence that TDF was very safe and effective for long-term use in the treatment of CHB patients. Minor adverse events such as nephrotoxicity or decreased bone density were occasionally reported[76]. Current international guidelines recommend TDF and ETV, which are the two most effective antiviral agents, as first-line treatments for compensated and decompensated liver diseases in chronic HBV infection.

MECHANISMS AND TREATMENT OF DRUG RESISTANCE

Drug resistance is one of the most important causes of antiviral treatment failure in patients with CHB infection. The emergence of HBV mutants during antiviral treatment leads to a rapid increase in viral replication, resulting in histological deterioration with ALT flares. Therefore, it is critical to detect early HBV mutants in the treatment of CHB patients to improve long-term prognosis of the disease.

Although HBV is a DNA virus, its replication is processed by reverse transcriptase through an RNA intermediate. Because HBV-DNA polymerase lacks a proofreading mechanism, mutations can occur if NAs incompletely suppress viral replication. Under the selective pressure of NAs, newly emerging mutant strains are selected as predominant species. Subsequently, compensatory mutations in the presence of the primary mutation may emerge at other regions of the polymerase gene, resulting in increased viral replication compared to wild-type HBV[77]. There are three factors that are involved in the development of antiviral resistance; viral fitness, potency, and genetic barrier to resistance of the NAs[78].

Resistance to NAs is dependent on the structure of the NA. Eight codons in HBV polymerase are known to be involved in the development of antiviral resistance: 169, 180, 181, 184, 202, 204, 236, and 250. There are five pathways leading to antiviral resistance[79]: (1) the rtM204V/I pathway for L-nucleosides (LAM, LdT and Clevudine); (2) the rtN236T pathway for alkyl phosphonates (ADV, TDF); (3) the rtA181T/V pathway, which is shared between the L-nucleosides and alkyl phosphonates; and (4) the D-cyclopentane/ETV pathway (rtL180M + rtM204V + I169T + T184S/G/C + S202C/G/I + M250I/V).

The general principles of antiviral therapy for NA resistance include the following: (1) confirmation of the patient's adherence to the drug; (2) detection of viral breakthrough and identification of genotypic resistance; and (3) consideration of combination therapy to prevent additional mutation caused by sequential NA therapy.

Lamivudine resistance

Although LAM is safe and tolerated in compensated and decompensated liver diseases due to HBV, it has limited clinical use in CHB patients due to a high rate of drug resistance. Previously, ADV alone or in combination with ongoing LAM therapy was used for the treatment of rtM204V/I mutant by LAM[56]. However, the rate of ADV resistance after direct switching from LAM to ADV in LAM-resistant patients was as high as 18% at year 1 and 65.6% at year 5, respectively[80,81]. On the other hand, rescue therapy switching to ETV at 1 mg daily for LAM-resistant patients was adopted, but genotypic ETV resistance and virological breakthrough developed in 51% and 43% of cases at year 5, respectively[63]. These results may be explained by cross-resistance shared by LAM and ETV. Thus, ETV monotherapy at a dose of 1 mg has not been further recommended as rescue therapy in LAM-resistant patients. TDF rescue therapy for ADV-resistant patients who were treated with ADV for 15 mo due to LAM-resistance, showed profound viral suppression of HBV-DNA below the detection limit of 400 copies/mL in 95% of cases[82]. Subsequently, a long-term study of TDF rescue monotherapy was conducted in patients with prior failure or resistance to different NA treatments[83]. Pretreatment consisted of either monotherapy with LAM, ADV, and sequential LAM-ADV therapy, or add-on combination therapy with both drugs. After a mean treatment duration of 23 mo, HBV-DNA was undetectable (< 400 copies/mL) in 79% of all patients. In addition, loss of HBeAg and HBsAg occurred in 24% and 3% of patients, respectively, suggesting that TDF monother-

Table 2 Recommended treatment for drug resistance

Resistance to	AASLD 2009	EASL 2012	APASL 2012
LAM	Add ADV or TDF Switch to Truvada[1]	Switch to TDF (add ADV if TDF is not available)	Add ADV Switch to TDF Switch to IFN-based therapy
LDT	Add ADV or TDF Switch to Truvada1	Switch to or add TDF (add ADV if TDF is not available)	Add ADV Switch to TDF
ADV	Add LAM Switch to Truvada[1] Switch to or add ETV	Switch to ETV or TDF Switch to TDF and add a nucleoside analogue if the patient has been treated with LAM	Add LAM, LDT or ETV Switch to TDF Switch to ETV plus TDF, if the patient has been treated with LAM or LDT
ETV	Switch to TDF or Truvada[1]	Switch to or add TDF (add ADV if TDF is not available)	Add TDF or ADV
TDF	Unmentioned	Add ETV, LDT, LAM or emtricitabine Switch to ETV if the patient has not been treated with LAM in the past	Unmentioned
MDR	Unmentioned	Combination of a nucleoside and a nucleotide	Unmentioned

[1]Truvada is a combination pill containing emtricitabine 200 mg and tenofovir 300 mg. LAM: Lamivudine; LDT: Telbivudine; ADV: Adefovir; ETV: Entecavir; TDF:Tenofovir; MDR: Multidrug resistance; IFN: Interferon.

apy induced a potent and long-lasting antiviral response in NA-experienced patients with previous treatment failure[82]. PEG-IFN as rescue therapy for LAM-resistant patients may be considered for compensated liver disease. Current international guidelines include recommendations regarding rescue therapy for NAs-resistant patients (Table 2). For LAM-resistant patients, switching to TDF and ADV add-on therapy are commonly recommended. IFN-based therapy for those patients is only recommended in the APASL guideline.

Adefovir resistance

ADV resistance is caused by rtN236T mutation in the D domain and rtA181T/V in the B domain of the HBV polymerase gene. A long-term follow-up study of ADV monotherapy for 48 wk in HBeAg-negative CHB showed ADV resistance in 0% at year 1 and 29% of patients at year 5[59], suggesting that long-term use of ADV could induce mutant strains. TDF has been proved to be effective in patients who were resistant to LAM and ADV. Although TDF shares partial resistance with ADV, it has been shown to have some antiviral effects in ADV resistance. In a recent study, TDF monotherapy was effective in patients with virological breakthrough or suboptimal response to ADV, but complete viral suppression was not found in patients with ADV-resistance, suggesting that a combination therapy of TDF with a NA should be considered in patients with ADV-resistance[84]. Because TDF possesses some degree of cross-resistance to ADV, the combination therapy of TDF plus LAM, LdT or ETV for patients with ADV-resistance may be more beneficial than TDF monotherapy to prevent further emergence of mutant strains. However, another study demonstrated that there was no significant difference in viral suppression between the combination of TDF plus emtricitabine and TDF alone in patients with CHB infection who had an incomplete response to ADV[85]. Further investigation is required.

Telbivudine resistance

LdT has relatively higher antiviral potency than LAM, however, prolonged treatment with LdT is limited by high rates of resistance due to a low genetic barrier. Studies reporting 1- and 2-year resistance rates to LdT were 5% and 25% in HBeAg-positive CHB patients, respectively[65]. A recent study revealed that LdT and ADV combination therapy led to significant decreases in serum HBV-DNA levels and normalization of ALT levels in patients with virological breakthrough or genotypic resistance to LdT[86]. Current international guidelines recommend add-on ADV or switching to TDF or Truvada in patients with LdT resistance.

Clevudine resistance

Clevudine is a nucleoside analogue with potent antiviral activity against HBV. Clevudine resistance in patients who have received clevudine therapy has been reported[70,87]. Although a long-term follow-up study of rescue therapy in clevudine-resistant patients has not been reported, the M204I mutation plays a key role in clevudine resistance and leads to virological breakthrough during long-term treatment. Drug susceptibility assays revealed that ADV and TDF were the most effective compounds against clevudine-resistant mutants. These results provide evidence of therapeutic options for clevudine-resistant patients. However, further clinical studies are required.

Entecavir resistance

ETV resistance occurs following a two-step mutation of HBV: (1) Pre-existing M204V and L180M by LAM; and (2) acquisition of the primary ETV resistance-encoding substitutions T184G and S202I or M250V. These mutations decrease ETV susceptibility and could give rise to virological rebound, leading to treatment failure[88].

After long-term ETV treatment in patients with LAM resistance, the rate of resistance was 51%[63]. This high resistance rate may be explained by rtM204V/I reducing the genetic barrier of ETV. Although the ETV resistance rate is not as high as other L-nucleoside analogues in naïve CHB, ETV rescue monotherapy at a dose of 1 mg for LAM-resistant patients should be considered for treatment failure due to virological rebound. Thus, add-on ADV to ETV may be beneficial in LAM-resistant patients to prevent further development of ETV resistance. Although clinical evidence regarding treatment for ETV resistance is insufficient to date, a recent study demonstrated the efficacy of TDF in ETV resistance[83]. Current international guidelines recommend add-on ADV or TDF or switching to TDF or Truvada in patients with ETV resistance.

Tenofovir resistance

TDF has been accepted as the most effective and safe antiviral nucleotide analogue for NA treatment-naïve or -failure patients to date. Recently, an open-label, long-term extension study of two previous phase Ⅲ studies which confirmed no resistance to TDF after 48-wk treatment[74,75] revealed that TDF monotherapy maintains effective suppression of HBV-DNA through 288 wk (6 years) of treatment with no evidence of TDF resistance[89]. However, virological breakthrough occurred infrequently and was associated with non-adherence to study medication in the majority of cases. According to current international guidelines, TDF is recommended as an essential antiviral agent for compensated or decompensated liver diseases irrespective of any resistance to NAs.

Multidrug resistance

In general, sequential NA monotherapy against HBV can promote selection of multidrug-resistant (MDR) strains of HBV. In particular, this frequently occurs with sequential treatment using NAs with similar characteristics (*e.g.*, LAM followed by ADV or ETV)[79]. Studies have shown that a combination therapy of TDF plus ETV in patients who had resistance to both LAM and ADV was effective[90,91]. A combination therapy of TDF plus ETV was shown to be effective in patients with MDR including LAM, ADV and ETV resistance. These results suggest that a combination therapy including TDF is better than other NA monotherapies or sequential therapy.

Currently, HBV drug-resistance mutation is mainly detected by direct sequencing and reverse hybridization. However, there are still limitations in identifying different substitutions present in the same viral variant with classic sequencing techniques[92]. Thus, it is necessary to find new tools to sequence large portions of the genome. Recently, a next generation sequencing (NGS) method has been developed to increase sequencing capacity while generating clonal sequences. The NGS method is processed through 3-step sequencing: library preparation, DNA capture and enrichment, and sequencing[93]. Newly developed virological tests may offer more optimized and tailored therapeutic strategies for treatment-naïve or -failure patients with chronic HBV infection. Current international guidelines do not recommend an appropriate treatment for MDR HBV caused by the anti-HBV NAs. However, based on pre-existing evidence, a combination of TDF and ETV is considered to be the most appropriate treatment for patients with MDR.

CONCLUSION

Although preventive vaccines and new antiviral agents against HBV have resulted in remarkable advances, chronic HBV infection is a still a difficult disease to control due to continuous viral replication from cccDNA in the nuclei of infected hepatocytes. Currently, there are two therapeutic approaches to control HBV replication: immunomodulating therapy and NAs. Despite extensive research, the exact roles of the immune response in controlling HBV infection or emerging HBV drug resistance are not completely understood. NAs therapy is considered an effective, safe and convenient therapy to control chronic HBV infection. However, the emergence of HBV mutant strains can lead to long-term treatment failure. The risk is very low or absent with ETV and TDF. Immune-based anti-viral strategies against HBV should be explored to achieve sustained viral control. International evidence-based guidelines have been developed to support physicians in managing CHB patients. However, it is difficult to initiate comprehensive therapy for MDR to NA as there is a lack of strong evidence to prove such issues. In addition, the currently available genotypic test, direct sequencing, still has limitations in identifying different substitutions present in the same viral variant. Therefore, the development of new virological tests identifying MDR may offer more optimized and tailored therapeutic strategies for treatment-naïve or -failure patients with chronic HBV infection. The factors influencing long-term treatment failure include patient compliance to drug, the emergence of HBV mutant strains, host genetic factors and the properties of each NA. It is critical to monitor viral load, serum HBsAg and ALT levels during antiviral therapy so that the therapeutic strategy can be continued or modified for patients with viral breakthrough or suboptimal response. Current practice guidelines stress the importance of profound and durable HBV viral suppression in the treatment of CHB patients. To this end, it is essential to choose a potent antiviral drug with a low risk of resistance for the initial treatment of CHB to achieve sustained virological response. TDF or ETV have a high genetic barrier and potent antiviral activity, and are widely accepted as first-line therapy for naïve patients at the present time. Furthermore, for patients with HBV mutant strains, combination therapy with NAs which have a high genetic barrier is more effective than sequential therapy of NAs with a low genetic barrier. Further studies are needed to explore the molecular mechanism of HBV mutations conferring resistance

to NAs and to achieve optimal therapeutic modalities for patients with treatment failure. Finally, new anti-viral strategies that can persistently suppress HBV replication and overcome drug resistance should be investigated for the treatment of patients with chronic HBV infection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease and vascular disease: State-of-the-art

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Abstract

Nonalcoholic fatty liver disease (NAFLD), the most common of chronic liver disease in Western Country, is closely related to insulin resistance and oxidative stress and includes a wide spectrum of liver diseases ranging from steatosis alone, usually a benign and non-progressive condition, to nonalcoholic steatohepatitis (NASH), which may progress to liver fibrosis and cirrhosis. NAFLD is considered the hepatic manifestation of the metabolic syndrome with which shares several characteristics, however recent data suggest that NAFLD is linked to increased cardiovascular risk independently of the broad spectrum of risk factors of metabolic syndrome. Accumulating evidence suggests that the clinical burden of NAFLD is not restricted to liver-related morbidity and mortality, with the majority of deaths in NAFLD patients related to cardiovascular disease and cancer and not to the progression of liver disease. Retrospective and prospective studies provide evidence of a strong association between NAFLD and subclinical manifestation of atherosclerosis (increased intima-media thickness, endothelial dysfunction, arterial stiffness, impaired left ventricular function and coronary calcification). A general agreement emerging from these studies indicates that patients with NASH are at higher risk of cardiovascular diseases than those with simple steatosis, emphasizing the role of chronic inflammation in the pathogenesis of atherosclerosis of these patients. It is very likely that the different mechanisms involved in the pathogenesis of atherosclerosis in patients with NAFLD have a different relevance in the patients according to individual genetic background. In conclusion, in the presence of NAFLD patients should undergo a complete cardiovascular evaluation to prevent future atherosclerotic complications. Specific lifestyle modification and aggressive pharmaceutical modification will not only reduce the progression of liver disease, but also reduce morbidity for cardiovascular disease improving overall prognosis and survival.

Key words: Intima-media thickness; Steatosis; Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis; Early atherosclerosis; Cardiovascular risk; Inflammation; Epicardic fat

Core tip: Nonalcoholic fatty liver disease (NAFLD) is emerging as an independent risk factor for the occurrence and progression of cardiovascular disease. In this review we have systematically analyzed the correlation between NAFLD and cardiovascular diseases (CVD) focusing on the different aspects of CVD (increased carotid intima media thickness, increased arterial stiffness, endhotelial dysfunction, impaired left ventricular function, coronary calcification, epicardic fat), on the clinical manifestations (obstructive sleep apnea syndrome, clinical consequences of microangiopathy), and on the underlying physiopathogenic mechanisms (insulin resistance, atherogenic dyslipidemia, inflammation, oxidative stress, adipokynes imbalance, coagulation

imbalance, increased assumption of fructose). Furthermore, we emphasized that NAFLD, by itself, is probably an independent risk factor for the occurrence of CVD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents an emerging public health problem worldwide, has reached epidemic proportions and, currently, is the most common of chronic liver disease in Western Country. NAFLD includes a wide spectrum of liver diseases ranging from steatosis alone, usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to liver fibrosis and cirrhosis. NAFLD is defined by the accumulation of liver fat > 5% per liver weight, in the presence of < 20 g/d for women and < 30 g/d for men of daily alcohol intake; viral or other causes of liver disease must be excluded[1-4]. Patients with NAFLD usually present no manifestation of liver disease and are often identified by elevation of liver enzymes or ultrasonographic evidence of liver steatosis[5].

NAFLD affects about 20%-30% of the general population and is closely related to insulin resistance and oxidative stress[4,6,7]. The reported prevalence of NAFLD varies widely depending on the population studied and definition used and ranges around 20%-25% in the general population, reaching values of 50% and 75% in obese subjects and 65%-90% in those affected by type 2 diabetes[8]. The prevalence was even higher (96%) in obese undergoing bariatric surgery[9]. Interestingly, children are also known to be at risk of developing NAFLD: the prevalence was about 3%-10% in lean subjects and about 53% in obese pediatric population. In the United States, 9-15 millions of people are affected by NASH (prevalence of 3%-5%) of whom 15%-20% may evolve to cirrhosis[10].

In addition to be at risk for NASH, cirrhosis and its complications, NAFLD patients are also at higher risk of systemic and cardiovascular diseases (CVD), including coronary heart disease and stroke[7]. NAFLD is considered the hepatic manifestation of the metabolic syndrome (MS), with which shares several characteristics, and despite the relationship between NAFLD and MS is now increasingly recognized, recent data suggest that NAFLD is linked to increased cardiovascular risk independently of the broad spectrum of risk factors of MS. Indeed, it is hypothesized that NAFLD is not merely a marker of cardiovascular disease but may also be involved in its pathogenesis[7,11-13] (Figure 1).

NAFLD, diagnosed either by ultrasonography or liver biopsy, has been associated with a higher prevalence of early asymptomatic cardiovascular alterations such as increased carotid intima-media thickness (IMT) and carotid atherosclerosis[13-15], coronary calcification[13,16], altered left ventricular geometry and early left ventricular diastolic dysfunction[17], and low coronary flow reserve[18]. In a recent study a higher prevalence of NAFLD was also shown in patients with atrial fibrillation[19]. These alterations have been partly associated with the severity of liver damage, defined by both lobular inflammation and fibrosis.

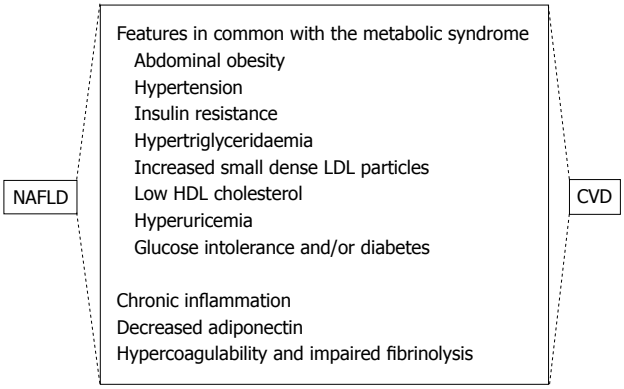

Figure 1 Common features in nonalcoholic fatty liver disease and cardiovascular disease. NAFLD: Nonalcoholic fatty liver disease; CVD: Cardiovascular disease.

Thus, accumulating evidence suggests that the clinical burden of NAFLD is not restricted to liver-related morbidity and mortality, with the majority of deaths in NAFLD patients related to cardiovascular disease and cancer and not to the progression of liver disease.

In the present review we present an overview on the state of the art of the latest discoveries about the correlation between NAFLD and CVD focusing on the different aspects of CVD (increased carotid intima media thickness, increased arterial stiffness, endothelial dysfunction, impaired left ventricular function, coronary calcification, epicardic fat), on the clinical manifestations [obstructive sleep apnea syndrome (OSAS), clinical consequences of microangiopathy], and on the underlying physiopathogenic mechanism (insulin resistance, atherogenic dyslipidemia, inflammation, oxidative stress, adipokynes imbalance, coagulation imbalance, increased assumption of fructose).

SURVIVAL STUDIES

Recent evidence suggests that NAFLD patients have a decreased survival as compared with general population, and cardiovascular disease dictates the outcome in NAFLD patients more frequently and to a greater extent than does the progression of liver disease[11]. Over 6-28 years of follow-up, several hospital-based studies have reported that patients with NAFLD (biopsy-proven or diagnosed by ultrasound) have a significantly higher all-cause and CVD-related than general population. Adams *et al*[20] in a population-based cohort study, found that 25% of related NAFLD death was linked to acute

myocardial infarction and only 13% to liver disease. In a study published in 2006, which included 212 consecutive patients with biopsy-proven NAFLD, it was found an increased mortality in NASH but not in simple steatosis. Moreover, this study showed that the higher mortality of NASH patients was primarily a result of CVD and, to a lesser extent, of liver-related causes[5]. Similarly, in a study of patients with biopsy-proven NAFLD with 21 years follow-up the main causes of death were CVD and malignancy[21]. Rafiq *et al*[22] in a long-term follow-up evaluation showed that death from coronary artery disease was the most common cause of death followed by malignancy; the liver-related death, including death from hepatocellular carcinoma, was the third most common cause of death. Furthermore, Söderberg *et al*[23] recently confirmed that NASH, but not simple steatosis, was associated with increased mortality from all causes during a 28 years follow-up; cardiovascular disease was the most frequent cause of death followed by extrahepatic cancer and, at third place, by liver-related causes. It should be noted, however, that these studies had a retrospective design and small cohort sizes and the adjustment for potential confounding metabolic factors was not performed[24]. Stepanova *et al*[25] in a combined large cohort of patients with biopsy-proven NAFLD confirmed that cardiovascular (CV) death remains the top cause of death in the NAFLD cohort. However, this retrospective study did not show any difference in CV mortality between NASH and non-NASH subtypes of NAFLD.

A series of prospective studies demonstrated that NAFLD was independently associated with increased risk either of non-fatal CVD events or CVD mortality (Table 1[26]). Targher *et al*[27] using age and sexed matched controls from a diabetes outpatient department, reported that the presence of NAFLD, diagnosed by ultrasound, was independently associated with an increased risk of incident non-fatal CVD events and CVD mortality over 6.5 years of follow-up. Large community and population-based studies showed similar results not only in European but also in Eastern countries[12,28-31]. Lazo *et al*[32] in 2011 and Stepanova *et al*[33] in 2012, using a database of over 11000 US participants found that NAFLD was independently associated with the risk of CVD, but, in contrast with the previous studies, not with increased risk in all-cause mortality. This study, however, was limited by the inclusion of mild hepatic steatosis within the control arm and by using only ultrasonography to exclude hepatic steatosis. Similarly, Kim *et al*[34] in the third NHANES study population found that ultrasonography - defined NAFLD was not associated with overall mortality but, interestingly, NAFLD with advanced fibrosis, was associated with the risk of overall mortality, of which the majority of deaths were due to CVD, even after adjustment for other common risk factors. Also this study had the same limitation of the two above (same database).

Additional long-term prospective and case-control studies are needed to demonstrate the independent risk of premature CVD and decreased survival in patients with well-defined NAFLD/NASH. In particular, understanding how modification of the progression of liver disease could affect and modify CVD progression and outcome is essential.

CAROTID INTIMA MEDIA THICKNESS

Increased IMT of carotid artery, considered a validated parameter of subclinical atherosclerosis and an independent predictor of stroke and myocardial infarction[35-38], and the presence of plaques as index of late atherosclerosis damage, have been used to characterize CVD risk of patients with NAFLD.

Retrospective studies

Several papers reported a strong association between increased IMT and NAFLD. One of the first studies was performed by Targher *et al*[39] in 2004. In this case-control study emerged that in NAFLD patients, all non-obese volunteers, with normal or moderately increased body weight, abdominal visceral fat accumulation was the key mediator for the relationship linking nonalcoholic hepatic steatosis to increased IMT.

Interestingly, many of these studies, conducted among different ethnic populations, showed that NAFLD predicted an increased carotid IMT independently from traditional risk factors, including insulin-resistance[15,40-48]. Fracanzani *et al*[15] in a paired-sample case-control study (125 patients with nonalcoholic fatty liver disease and 250 controls), without a prior diagnosis of diabetes, hypertension, and cardiovascular disease, matched for sex, age, and body mass index found a significant difference in mean values of IMT (0.89 ± 0.26 mm and 0.64 ± 0.14 mm, $P = 0.0001$) and prevalence of plaques [26 (21%) and 15 (6%), $P < 0.001$] between the two groups. In this study independent risk predictors of IMT higher than 0.64 mm (the median value of normal subjects) were presence of steatosis (OR = 6.9), age (OR = 6.0), and systolic blood pressure (OR = 2.3). Aygun *et al*[49] in a population of 40 age-matched-control and biopsy proven NAFLD patients reported that metabolic syndrome and increased IMT were more prevalent in the NAFLD cohort. Kim *et al*[42] in a case-control study stratified by gender concluded that NAFLD was independently associated with carotid atherosclerosis in patients with metabolic syndrome.

Salvi *et al*[50] in the Cardio-GOOSE (Cardio-Gambettola ObservatOry liver Steatosis Estimation) study, a population-based cohort study finalized to evaluate the relationship between NAFLD and subclinical vascular damage, showed that NAFLD was associated with the metabolic syndrome in 48% of cases. IMT values were strongly related to the number of metabolic syndrome factors. In fact no significant differences in IMT were found between controls and patients with NAFLD and no manifestation of metabolic syndrome (0.77 +/- 0.15 mm *vs* 0.76 +/- 0.14 mm). Conversely, in patients with NAFLD and associated metabolic syndrome, IMT values were significantly higher than in patients with NAFLD

Table 1 Prospective and retrospective studies of the risk of cardiovascular mortality in patients with nonalcoholic fatty liver disease diagnosed by liver biopsy and/or by ultrasonography

Ref.	Study design	NAFLD patients	Diagnosis of NAFLD	Mean duration follow-up (yr)	Result
Matteoni *et al*[26], 1999	Retrospective, Hospital-based	132	Histology	18.0	No adjustment made. Higher all-cause, liver-related mortality but no difference in CVD mortality (NASH *vs* simple steatosis)
Dam-Larsen *et al*[21], 2004	Retrospective, Hospital-based	109	Histology	16.7	Analysis performed by gender. No difference in all-cause and liver-related or CVD mortality (simple steatosis *vs* general population)
Adams *et al*[20], 2005	Retrospective community-based	420	US and/or histology	7.6	Matched for gender, age and country Higher all-causae, liver-related and CVD mortality (CVD is the second cause of death by frequency) in NAFLD patients (especially cirrhosis)
Targher *et al*[12], 2005	Prospective case-control, Hospital-based	248/2103[1]	US	5.0	NAFLD was independently associated with increased nonfatal CVD and CVD mortality
Ekstedt *et al*[5], 2006	Retrospective, Hospital-based	129	Histology	13.7	Matched for gender, age and country Higher all-cause, liver-related and CVD mortality (NASH but no simple steatosis *vs* general population)
Hamaguchi *et al*[28], 2007	Prospective, community-based	312/1637[2]	US	5.0	NAFLD was independently associated with increased risk of nonfatal CVD events
Targher *et al*[27], 2007	Prospective, Hospital-based	1421/2103[1]	US	6.5	NAFLD was independently associated with increased risk of nonfatal CVD events and CVD mortality
Haring *et al*[29], 2009	Prospective, community-based	2490/4160[3]	US and altered GGT	7.3	NAFLD was independently associated with increased risk of all-cause and CVD mortality in men
Rafiq *et al*[22], 2009	Retrospective, Hospital-based	173	Histology	13.0	No adjustments made Higher liver-related mortality, but no difference in overall mortality (NASH *vs* simple steatosis)
Söderberg *et al*[23], 2010	Retrospective, Hospital-based	118	Histology	18.0	Matched for gender, age and year Higher all-cause, liver-related and CVD mortality (NASH but no simple steatosis *vs* general population)
Lazo *et al*[32], 2011	Prospective, population-based (third NHANES study population 1988-1994)	2089/11371[4]	US	14.3	Independent increased risk of CVD but no increased risk in all-cause, liver-related and CVD mortality
Stepanova *et al*[33], 2012	Prospective, population-based (third NHANES study population 1988-1994)	2492/11371[4]	US	14.3	Independent increased risk of CVD but no increased risk in all-cause, liver-related and CVD mortality
Treeprasertsuk *et al*[31], 2012	Prospective, community-based	309	US, CT, MRI, and or Histology	11.5	Higher 10-Year CVD risk (NAFLD *vs* general population)
Kim *et al*[34], 2013	Prospective, population-based (third NHANES study population 1988-1994)	4083/11154[4]	US and NAFLD Fibrosis Score	14.5	US-Defined NAFLD was not associated with overall mortality. NAFLD with advanced fibrosis was independently associated with overall mortality (majority of deaths were due to CVD)
Stepanova *et al*[25], 2013	Retrospective, Population-based	289	Histology	6.25	No adjustments made Higher risk of liver-related mortality in NASH than non-NASH. NAFLD and type II diabetes highest risk for overall and liver-related mortality

[1]Patients with type 2 diabetes; [2]Participants of health checkup program; [3]West Pomerania population; [4]United Stated adult population. US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; CVD: Cardiovascular disease; NAFLD: Nonalcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

alone (0.85 +/- 0.16 mm, $P < 0.005$)[50]. From a large population based survey of over 8500 middle aged and elderly Chinese population, Huang *et al*[46] reported that NAFLD was associated with elevated IMT independently of the conventional risk factors. Thakur *et al*[51] in a case-control study in non-diabetic population reported that NAFLD seemed to be an independent predictor of having high average IMT, independently of obesity and metabolic syndrome. Notably, this is the first study in Asian Indians demonstrating the association.

The association between NAFLD and increased carotid IMT was independent of the metabolic syndrome in at least four studies[44-47].

Increased IMT and liver damage

It has been reported that subclinical cardiovascular disease was significantly associated with the severity of liver disease. Targher *et al*[52] compared carotid IMT in 85 consecutive patients with biopsy-proven NAFLD and 160 age-, sex-, and BMI-matched healthy control

subjects and found that the severity of liver histopathology in NAFLD patients was strongly associated with early carotid atherosclerosis, independently of classical risk factors, insulin resistance, and the presence of metabolic syndrome, suggesting that the severity of liver damage could play a role in atherosclerosis development. Băloşeanu *et al*[53] reported that the severity of liver histopathological lesions among NAFLD patients was strongly associated with increased IMT. This study, however, had a limitation because it was performed in a small sample of NAFLD subjects. Also Kucukazman *et al*[54] in a recent study found that the mean carotid IMT was significantly increased in patients with NAFLD and showed a significant positive cancel weak correlation between ultrasonographic steatosis grade and mean IMT ($r = 0.376$, $P < 0.001$).

IMT and type 2 diabetes

At least four studies examined the link between NAFLD, type 2 diabetes and subclinical atherosclerosis damage. It is well known that NAFLD is extremely common in people with type 2 diabetes and is mainly associated with uncontrolled diabetes. Targher *et al*[55] studied a population of 200 diet-controlled type 2 diabetic subjects, reported that NAFLD patients had a markedly greater carotid IMT and that the increase of carotid IMT was largely explained by HOMA-estimated insulin resistance. In contrast IMT values were found to be significantly higher in diabetic patients regardless of the degree of steatosis by Cakir *et al*[56]. Similarly other two studies performed in diabetic population reported that hepatic steatosis was not associated with carotid atherosclerosis and suggested that the association of hepatic steatosis and cardiovascular disease might be just an epiphenomenon[57,58].

Pediatric population

One of the first evidence of the association between fatty liver and atherosclerosis were the autoptic findings in 817 children who died of external causes showing that fatty liver was present in 15% of the children, mild atherosclerosis in 21% and moderate to severe atherosclerosis in 2%. Atherosclerosis was found more common in children with fatty liver (30%) than those without fatty liver (19%). Interestingly, growing evidence suggests a strong relationship between early carotid atherosclerosis and NAFLD, although longitudinal studies are needed to clarify the extent to which pediatric NAFLD and its severity influence long-term cardiovascular outcomes in the general population[59-65].

In conclusion, considering the large number of publications on the relationship between IMT and vascular damage, carotid screening for NAFLD might be beneficial for assessment of future atherosclerotic complications.

ARTERIAL STIFFNESS

Measurement of biomechanical properties of arteries has become an important surrogate outcome used in epidemiological and interventional cardiovascular research. A loss of arterial elastic properties results in a range of linked pathophysiological changes in the circulation including increased pulse pressure, left ventricular hypertrophy, subendocardial ischaemia, vessel endothelial dysfunction and cardiac fibrosis[66]. Arterial stiffness, determined by measurement of pulse wave velocity, has been shown to be an independent indicator of symptomatic cardiovascular disease[67]. Carotid and femoral pulse wave velocity has been considered the noninvasive gold standard measure of arterial stiffness[50]. Brachial-ankle pulse wave velocity is also widely used to screen for atherosclerotic vascular damage[13,67]. Several recent studies reported a strong association between NAFLD and impaired arterial elastic properties. In the GOOSE study Salvi *et al*[50] showed that NAFLD may play an independent role in determining arterial stiffness. Vlachopoulos *et al*[67] in a case-control study with biopsy-proven NAFLD reached similar results. Huang *et al*[46] in the study including 8632 Chinese men aged $\geqslant$ 40 years showed that brachial-ankle pulse wave velocity was increased in NAFLD independently of conventional cardiovascular disease risk factors and metabolic syndrome. In another study of the same author performed in adolescents it was found that NAFLD was only associated with increased arterial stiffness in the presence of the "high risk" metabolic cluster, suggesting that arterial stiffness related to the presence of NAFLD was predicted in adolescents by the presence of an adverse metabolic profile[68]. Kim *et al*[69] in a case-control study indicated that the presence and the degree of NAFLD were associated with arterial stiffness, measured by brachial-ankle pulse wave velocity, in non-hypertensive, non-diabetic individuals, especially in those without metabolic syndrome. Similarly, Lee *et al*[70] showed that arterial stiffness, was independently associated with the risk for NAFLD regardless of classical CVD risk factors.

Işilak *et al*[71] and Fotbolcu *et al*[72] reported that elastic properties of aorta were abnormally changed in patients with NAFLD, probably related to or associated with insulin resistance[71,72]. In contrast, Catena *et al*[73] revealed that in essential hypertensive patients without additional cardiovascular risk factors, NAFLD was associated with insulin resistance but not with increased arterial stiffness.

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is one of the earliest markers of atherosclerosis. Recent studies showed an association between NAFLD and endothelial dysfunction, assessed by brachial artery flow mediated dilatation (FMD). In 2005 Villanova *et al*[74] reported that FMD was significantly reduced in NAFLD population; in this case-control study NAFLD, diagnosed by liver biopsy or by ultrasound, predicted a reduced FMD after adjusting for age, sex, BMI and insulin resistance. Senturk *et al*[75] also reported that endothelial dysfunction was worse in NASH compared to simple steatosis and controls suggesting that inflam-

mation in the liver plays a role.

Mohammadi *et al*[44] in a study performed in 2011, reported that pure NAFLD without metabolic syndrome in middle-aged subjects was strongly associated with morphological (increased carotid IMT) and physiological flow-mediated changes.

In a cohort of never treated uncomplicated hypertensive outpatient subjects the presence of NAFLD was associated with physiological FMD changes and insulin-resistance[76]. Thakur *et al*[51] in a case-control study reported that in a cohort of Asian Indians subjects NAFLD is significantly associated with subclinical atherosclerosis and endothelial dysfunction. Interestingly, these results were independent from the classical cardiovascular risk factors, *i.e.*, obesity and metabolic syndrome. In another observational case-control study the measurement of brachial artery FDM was significantly lower in patients with NAFLD group compared to control group suggesting that NAFLD, and in particular NASH, is associated with endothelial dysfunction, independently from metabolic syndrome[48]. Similar results were reported by Kucukazman *et al*[54]. In addition plasma levels of fetuin-A, which is known to inhibit insulin signalling and recently emerged as a biomarker for diabetes risk, was found independently associated with endothelial dysfunction and subclinical atherosclerosis in NAFLD patients[77].

IMPAIRED LEFT VENTRICULAR FUNCTION

At least seven studies reported that NAFLD was associated with impaired left ventricular diastolic dysfunction, alteration in energy metabolism and disturbance of cardiac rhythm (Table 2). Goland *et al*[78] found that patients with NAFLD, in the absence of morbid obesity, hypertension, and diabetes had early features of left ventricular diastolic dysfunction and mildly altered left ventricular (LV) geometry. Lautamäki *et al*[79] found that in patients with type 2 diabetes and coronary artery disease, liver fat content was a novel independent indicator of myocardial insulin resistance and reduced coronary functional capacity. Fallo *et al*[80] in a study conducted in a cohort of never-treated essential hypertensive patients with nonalcoholic fatty liver disease, reported that NAFLD was significantly associated with insulin resistance and abnormalities of left ventricular diastolic function, suggesting a concomitant increase of metabolic and cardiac risk. On the contrary, Perseghin *et al*[81] reported that in newly diagnosed individuals with fatty liver no impaired LV morphological features and systolic and diastolic functions were present but they had abnormal LV energy metabolism that correlated with higher mediastinal visceral fat. In a recent study in which 50 consecutive patients with type 2 diabetes were enrolled it was observed that in patients with NAFLD LV morphology and systolic function were preserved but early features of LV diastolic dysfunction were present[17].

Hallsworth *et al*[82] in a cohort of nineteen adults with NAFLD age-, sex-, and BMI-matched to healthy controls without liver or metabolic diseases, reported that adults with NAFLD had significantly thicker left ventricular wall at systole (14 ± 3 mm *vs* 12 ± 2 mm, $P < 0.01$) and diastole (8 ± 1 mm *vs* 7 ± 1 mm, $P < 0.01$) than those without fatty liver and showed decreased longitudinal shortening (14% ± 3% *vs* 17% ± 3%, $P < 0.01$). This case-control study concluded that significant changes in cardiac structure and function are evident in adults with NAFLD in the apparent absence of metabolic changes or overt cardiac disease.

Interestingly, a recent study demonstrated that NAFLD was associated with an increased risk of incident atrial fibrillation (AF), (OR = 4.49, 95%CI: 1.6-12.9, $P < 0.005$). Adjustments for age, sex, hypertension and electrocardiographic features (left ventricular hypertrophy and PR interval) did not attenuate the association between NAFLD and incident AF (adjusted OR = 6.38, 95%CI: 1.7-24.2, $P = 0.005$)[19].

CORONARY CALCIFICATION

Computed tomography examination of the coronary arteries can detect calcification indicative of arterial disease in asymptomatic people which has been identified as a surrogate marker of subclinical atherosclerosis[83]. Coronary artery calcification (CAC) was considered an independent marker of CVD risk[84]. Akabame *et al*[85] in a case-control study, evaluating vulnerable coronary plaques and steatosis, by multi-slice computed tomography, showed that liver fat was significantly correlated with lipid core plaques. Adjusted odds ratio of NAFLD for remodeling lesions was 2.41 (95%CI: 1.24-4.67; $P = 0.009$), and those for lipid core lesions 2.29 (95%CI: 1.15-4.56; $P = 0.018$)[85]. A population study performed in 2007, evaluating 371 non-diabetic, asymptomatic middle-aged men free of known coronary heart disease, showed that the presence of hepatic steatosis was associated with a greater prevalence of risk factors for atherosclerosis, with a higher 10-year total coronary heart disease risk (calculating by Framingham Risk Score) and was independently associated with CAC in subjects with metabolic syndrome[86].

Three studies published in 2010 reported similar results. Assy *et al*[87] found that patients with NAFLD, even without metabolic syndrome, are at high risk for atherosclerosis and had a higher prevalence of coronary calcification. In a retrospective study, Chen *et al*[88] reported that besides traditional risk factors, such as male gender, increased age, and DM, NAFLD was also associated with moderate to high risk of coronary disease and with the severity of CAC. Jung *et al*[89] found, in a cohort of 1218 Korean subjects, that NAFLD predicted coronary calcification. Individuals with combined elevated ALT (> 30 U/L) and ultrasound diagnosed hepatic steatosis had higher odds of a CAC score > 100 compared to controls and individuals with elevated ALT or steatosis alone. However it is not possible to understand from the paper whether the patients with increased ALT had NASH.

Table 2 Studies of the impaired left ventricular function and disturbance of rhythm in patients with nonalcoholic fatty liver disease

Ref.	Study design	Study population	Study size	Diagnosis of NAFLD	Cardiac parameters examined	Results
Left ventricular function						
Goland *et al*[78], 2006	Retrospective, Case-control study, Hospital-based	NAFLD *vs* control	38 NAFLD 25 non-NAFLD	US or histology	Complete echocardiographic study, TDI, peak velocities of E and A diastolic filling, *E/A* ratio, *Vp*, E', and S' of mitral annulus	NAFLD patients had increased thickening of the intraventricular septum and posterior, lower E diastolic filling velocity, lower *E/A* ratio, longer, lower *Vp* and lower E' No differences were found according to LV systolic function
Lautamaki *et al*[79] 2006	Retrospective, Case-control study, Hospital-based	Patients with type 2 diabetes and CVD	28 low liver fat 27 high liver fat Matched for age, BMI, fasting glucose	H-MRS	Positron emission tomography. Myocardial perfusion was measured with[^{15}O]H_2O and myocardial and skeletal muscle glucose uptake with 2-deoxy-2-[^{18}F]fluoro-D-glucose during hyperinsulinemic euglycemia	Liver fat content is independently associated with impaired myocardial metabolism
Fallo *et al*[80] 2009	Retrospective, Case-control study, Hospital-based	Never-treated essential hypertensive patients	48 NAFLD 38 non-NAFLD Matched for sex, age and blood pressure levels	US	Complete echocardiographic study: TDI, peak velocities of E and A diastolic filling, *E/A* ratio, *Vp*, E', and S' of mitral annulus	NAFLD patients had increased prevalence of left ventricular hypertrophy , diastolic dysfunction that increased according to the degree of NAFLD
Perseghin *et al*[81] 2008	Retrospective, Case-control study, Hospital-based	Young non-diabetic men	21 fatty liver 21 non fatty liver Matched for age, BMI, blood pressure levels, lipid values	H-MRS	MRI and MRS	No difference in morphological parameters of the LV, systolic and diastolic functions NAFLD patients had reduced values of PCr/ATP ratio
Bonapace *et al*[17] 2012	Retrospective, Case-control study, Hospital-based	Patients with type 2 diabetes	32 NAFLD 18 non-NAFLD Matched for age, sex, BMI, waist circumference, hypertension, smoking, diabetes duration, microvascular complication status, medication use	US	Complete echocardiographic study, TDI, peak velocities of E and A diastolic filling, *E/A* ratio, *Vp*, E', and S' of mitral annulus	NAFLD patients had lower E', tissue velocity, higher E-to-e' ratio, higher time constant of isovolumic relaxation, higher LV-end diastolic pressure (EDP), higher LV EDP/end diastolic volume No difference in morphological parameters of the LV, systolic functions
Hallsworth *et al*[82] 2013	Retrospective, Case-control study, Hospital-based	NAFLD and healthy controls	19 NAFLD 19 non-NAFLD Matched for age, sex, BMI, weight, and body surface area *vs* control	H-MRS	MRI and MRS	NAFLD patients had thicker left ventricular walls at systole and diastole, decreased longitudinal shortening, higher concentric remodelling. No difference in PCr/ATP ratio
Disturbance of cardiac rhythm						
Targher *et al*[19] 2013	Prospective, Hospital-based	Type 2 diabetes	281 NAFLD 119 non-NAFLD Adjustments for age, sex, hypertension and electrocardiographic features	US	12-lead electrocardiogram	NAFLD was associated with an increased risk of incident AF

A: Late; BMI: Body mass index; E: Early; E': Early diastolic velocity; H-MRS: Proton magnetic resonance spectroscopy; cardiac MRI: Magnetic resonance imaging; MRS: 31P-MR spectroscopy; NAFLD: Nonalcoholic fatty liver disease; PCr/ATP ratio: Phosphocreatine/adenosine triphosphate ratio; S': Systolic velocity; TDI: Tissue Doppler imaging; US: Ultrasonography; Vp: Flow propagation velocity; AF: Atrial fibrillation.

Kim *et al*[16] in the largest population-based study published in 2012 showed a strong relationship between NAFLD and CAC; 4123 cases and controls from Seoul were enrolled. The study reported that NAFLD predicted a CAC score > 10; computed tomography (CT) examination revealed that visceral adiposity attenuated but did not eliminate the

relationship between NAFLD and CAC (adjusted OR = 1.32 (95%CI: 1.12-1.57)[16]. These recent evidences suggested that detection of NAFLD could signal the existence of an increased coronary artery disease risk independent of visceral adiposity.

Only two studies showed no significant association between NAFLD and coronary calcification, probably one for the different selection of patients[57] and the other for the small series of patients (70 subjects)[90].

OBSTRUCTIVE SLEEP APNEA SYNDROME

OSAS is a common medical condition that affects about 4% of general population with higher prevalence among obese subjects (incidence 25%-35%)[91]. Besides obesity, age, male, sex, smoke and alcohol intake are risk factors for OSA. OSAS is a form of sleep disordered breathing, which is characterized by repetitive complete and/or partial collapses (apnea and hypopnea respectively) of the upper airways. OSAS is characterized by episodes of chronic intermittent hypoxia and sleep fragmentation, with increase in sympathetic activity and promotion of oxidative stress, pro-inflammatory cytokine production, platelet aggregation, endothelial dysfunction and metabolic dysregulation. All these mechanisms provide the pathophysiological basis for the increased risk of CVD observed in these patients[92].

OSAS has been identified as an independent comorbid factor in cardiovascular and cerebrovascular diseases, and physiopathological links may exist with onset and progression of heart failure. It has also been found an association between OSAS and the manifestations of the metabolic syndrome, in particular with obesity[91], and with arterial stiffness, endothelial dysfunction and carotid IMT[93]. The disease is classified as mild, moderate and severe based on the number of apneas and/or hypopneas per hour of sleep, known as the apnea-hypopnea index. This is assessed by polysomnography (PSG) or other forms of sleep monitoring[94]. Both OSAS and metabolic syndrome, of which NAFLD represents the hepatic manifestation, may exert negative synergistic effects on the cardiovascular system through multiple mechanisms (*e.g.*, hypoxemia, sleep disruption, activation of the sympathetic nervous system, and inflammatory activation). It has been found that continuous positive airway pressure therapy for OSAS provides an objective improvement in symptoms and cardiac function, decreases cardiovascular risk, improves insulin sensitivity and adiponectin level, reduces adipocytokines (leptin and resistin) levels, and normalized biomarkers[95].

Recent experimental evidence connected chronic intermittent hypoxia with insulin-resistance and reported that intermittent hypoxia concurred to the pathogenesis of NAFLD. Moreover, in NAFLD patients, the presence of OSAS resulted linked to the progression of NAFLD to NASH[91].

NAFLD AND MICROANGIOPATHY

It has been reported that NAFLD may also be associated with micro-vascular changes in other organs that have direct or indirect influence on CVD and may accelerate the presentation of CVD. For example, patients with NAFLD, in a type 2 diabetic population, had significant higher age- and sex-adjusted prevalence rates of both non-proliferative (39% *vs* 34%) and proliferative/laser treated retinopathy (11% *vs* 5%) and of chronic kidney disease (15% *vs* 9%) than counterparts without NAFLD. Furthermore, in this study, NAFLD was associated with an increased incidence of chronic kidney disease, independent of classical risk factors[96]. At least three studies showed an association between retinal vascular changes and NAFLD. Băloşeanu *et al*[53] in 10 biopsy-proven NAFLD patients, found a connection between the degree of NAFLD and the retinal vascular changes in those with carotid atherosclerosis. Josef *et al*[97] analyzing a cohort of non-diabetic and non-hypertensive patients, found that fatty liver (OR = 2.5; $P < 0.01$), IMT (OR = 2.3; $P < 0.001$), and retinal changes (OR = 1.5; $P < 0.01$) were strongly associated with CAD independent of metabolic syndrome (OR = 1.2; $P < 0.05$). Also Targher *et al*[98] suggested that patients with NAFLD have an increased prevalence of chronic kidney disease, known risk factor for CVD. An increasing number of studies showed that NAFLD may be an independent risk factor for chronic kidney disease[96,98-108]. In several retrospective studies the prevalence of chronic kidney disease defined as eGFR < 60 mL/min/1.73 m^2 or proteinuria- and/or microalbuminuria were significantly higher among NAFLD patients compared with those without[100,101,103]. Interestingly, the majority of these studies reported that NAFLD, and in particular NASH, was independently associated with chronic kidney disease /microalbuminuria even after adjusting for traditional risk factors, *i.e.*, age, sex, BMI, hypertension, diabetes, smoking and hyperlipidemia[100,102,104,109]. However some studies defined NAFLD according to elevated of GGT or ALT, and thus they need to be interpreted with caution due to the relative lack of diagnostic accuracy. In addition Neri *et al*[110] reported that, independently related with the traditional risk factors for cardiovascular disease, nonspecific inflammation and oxido-reductive imbalance may play an important role in the progression of NAFLD and atherosclerotic disease in hemodialysis patients.

In conclusion NAFLD, in particular biopsy proven NASH, was associated with a greater prevalence of microvessel damage, in particular chronic kidney disease.

EPICARDIC FAT

Ectopic fat accumulation within and around organs such as the heart and the liver are linked to an increased cardiovascular risk[111,112]. The epicardial fat, is considered the true visceral fat and the best representation of fatty heart[113]. Epicardial fat displays peculiar biomolecular

and anatomic characteristics, and its direct contiguity to myocardium reflects the intra-myocardial fat accumulation. Epicardial adipose tissue might secrete vasoactive products that regulate coronary arterial tone, in addition various studies have highlighted the potential importance of adipose tissue in relation to inflammatory burden in cardiovascular diseases. In fact epicardial fat has been demonstrated to be a source of several pro-inflammatory and proatherogenic cytokines that could influence the cardiac function[114-116]. It has been reported that epicardial fat also correlates with several cardiac comorbidities, including coronary artery disease, left ventricular dysfunction, atrial fibrillation; moreover a pivotal role of body fat has been suggested in the development and progression of both diastolic and systolic heart failure[117,118].

Epicardial fat has been found clinically related to abdominal visceral adiposity, coronary artery disease, subclinical atherosclerosis and metabolic syndrome[119-121]. In particular Lai *et al*[122] reported graded increases in fasting glucose, insulin resistance, and alanine transaminase levels across higher tertiles of epicardial fat thickness. In addition the measurement, by magnetic resonance imaging, of epicardial fat, as well as the degree of hepatic steatosis, correlates with abdominal adiposity and hypertriglyceridemia. A good relation between epicardial fat thickness and the degree of fatty liver severity was described in adult and pediatric patients with NAFLD and obesity[65,123], and with early atherosclerotic damage[45].

The imaging tools used to study and quantify epicardial fat were cardiac CT and magnetic resonance imaging (MRI). Recently, Lai *et al*[122] showed that ultrasonographic measurement of EAT thickness highly correlated with that measured with CT.

GENETIC

In the last few years, growing evidence indicates that in all diseases, including cardiovascular diseases, environment and genetic factors cooperate in inducing disease manifestation. Data arising from genome-wide (GWAS) or candidate association studies identified single nucleotide polymorphisms (SNPs) in genes involved in metabolic homeostasis, inflammation, oxidative stress and fibrogenesis, as associated with NAFLD and its severity. Patatin-like phospholipase-3 (PNPLA3)/adiponutrin, rs738409 C.G SNP, remains the most validated risk gene in this setting[124]. A recent study by Valenti *et al*[125] showed that PNPLA3 variant was directly related to hepatic apoptosis in NAFLD. According to these data, it is plausible to hypothesize that PNPLA3 genotype, by regulating apoptotic activity, may be also involved in the pathogenesis of atherosclerosis and might modulate vascular damage. Recently, Petta *et al*[126] showed that PNPLA3 GG genotype was associated with higher severity of carotid atherosclerosis in younger patients with NAFLD. PNPLA3 gene variants might increase lipid storage in the arterial vessels and could also induce release of ICAM-1, an endothelium-derived inflammatory marker that has been associated with myocardial infarction and stroke[127].

It was also hypothesized that the same pathways generating liver disease might also be involved in vascular damage.

RELATION BETWEEN NAFLD AND ATHEROSCLEROSIS INDEPENDENTLY OF KNOWN RISK FACTORS: POSSIBLE MECHANISMS

Growing evidence suggests that patients with NAFLD are at higher risk of cardiovascular disease than those without NAFLD, even when their primary disease (diabetes, hypertension…) has a strong potential for atherosclerosis[9,11,13].

Several mechanisms can be involved in atherosclerosis acceleration in patients with NAFLD, including genetic predisposition, insulin resistance and atherogenic dyslipidemia, oxidative stress, chronic inflammation, reduced levels of the adiponectin (an adipocytochine with antiatherogenic and antidiabetic properties), and altered production of pro and anticoagulant factors. All these mechanisms are present at the same time (Figure 2)[11,128].

The association between early atherosclerosis and NAFLD has been extensively described and summarized in Table 3 in which are reported variables independently associated with IMT in adult subjects[7,13,15,25,41-44, 46,47,49-52,55,58, 110,125,129-133].

INSULIN RESISTANCE

Normally insulin inhibits adipose tissue lipolysis and hepatic VLDL secretion by the liver, and on the contrary, insulin resistance promotes fatty acid accumulation in the liver which in turn causes an increase in insulin resistance responsible for a reduced suppression of endogenous liver glucose production. Given the very close relationship between NAFLD and insulin resistance[134,135], which is present in more than 90% of patients with NAFLD, it is still debated which one of the two is the earlier event. In the presence of overweight/obesity and of increased circulating insulin, due to insulin resistance, there is increased lipolysis of adipose tissue which leads to increased flux of free fatty acid (FFA) to the liver from all lipid depots, including ectopic fat. In addition hepatic lipid accumulation occurs also because of dietary chilomicrons and *de novo* intrahepatic lipogenesis. Further accumulation of lipids in the liver is, in turn, responsible for insulin resistance worsening with a consequent vicious circle. In addition, besides the augment of FFA, the increased release of different molecules from visceral adipose tissue, including inflammatory cytokines, leads to adipose tissue inflammation which aggravates insulin resistance and facilitates atherosclerosis development and cardiovascular disease. Experimental evidences indicate that activation of proinflammatory pathways occurs mainly through the nuclear

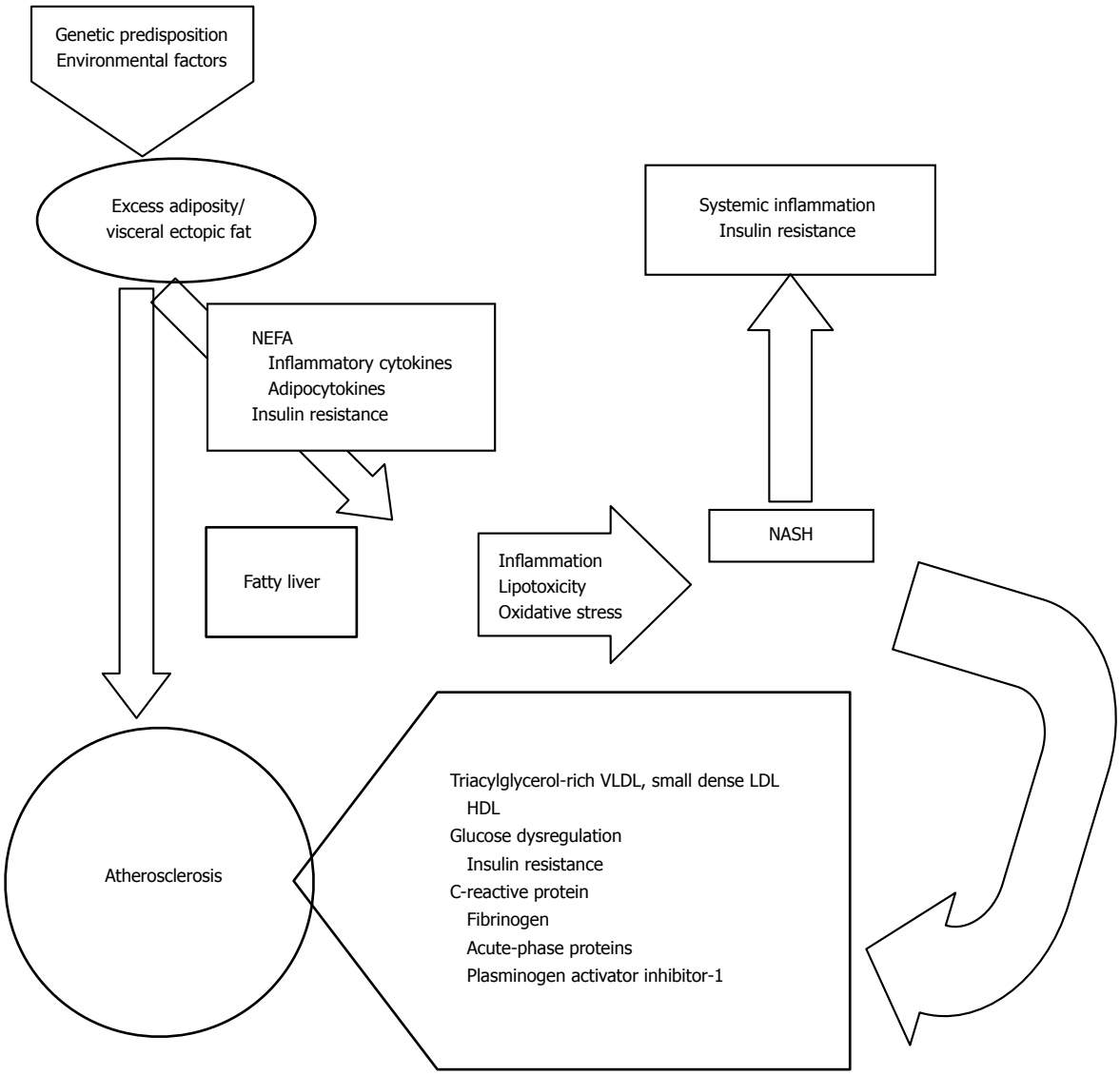

Figure 2 Mechanisms potentially responsible for atherosclerosis in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. NASH: Non-alcoholic steatohepatitis.

factor κB (NF-κB) and the c-Jun N-terminal kinase (JNK) pathways which cooperate in inducing insulin resistance which, in association with several factors, including chronic inflammation, atherogenic dyslipidemia, hypercoagulation and hypofibrinolysis, and liver involvement either as target of the metabolic abnormalities or as producer of proatherogenic molecules, triggers arterial damage leading to accelerated atherosclerosis. It is possible that in lean subjects, which are roughly 10%-15% of patients with NAFLD, the insulin resistance and chronic inflammation of visceral adiposity is at least initially replaced by insulin resistance in skeletal muscle[11].

ATHEROGENIC DYSLIPIDEMIA

Typical atherogenic dyslipidemia is frequently detected in NAFLD, characterized by high triglycerides and low HDL-C which, as above reported, together with insulin resistance and visceral adiposity play a major role in accelerated atherosclerosis[136-138]. A high level of low density lipoprotein (LDL) cholesterol, in particular of small dense LDL which are more atherogenic than type A LDL particles, is frequently detected in patients with NAFLD as well as increased levels of oxidized LDL, which are highly atherogenic. Moreover, in a recent large, multiethnic, gender balanced cohort, NAFLD was associated with the atherogenic dyslipidemia phenotype (higher fasting serum triglycerides and lower serum HDL) in a dose dependent fashion, suggesting a possible independent pathophysiologic role between NAFLD and dyslipidemia[139]. The main alteration believed to play a key role in atherosclerosis is the overproduction of larger TG-rich VLDL particles from the liver. Furthermore, as described in the insulin resistance paragraph, in the presence of increased adipose tissue there is an increased flux of FFA from adipose tissue to the liver where are re-esterified into triglycerides with consequent increased VLDL secretion into the blood stream. In addition, increased lipid

Table 3 Studies on the association between non-alcoholic fatty liver disease and carotid intima-media thickness in adults: evaluation of the independent predictors for intima-media thickness increase

Ref.	cases *n*	Cohort	NAFLD diagnosis by	Independent predictors for IMT
Brea *et al*[40] 2005	80	NALFD and matched population controls	US	NAFLD, age, serum ferritin
Volzke *et al*[41] 2005	1261 + 2961	German population	US	Hepatic steatosis
Lonardo *et al*[129] 2006	449	Population/NAFLD	US	Age
Targher *et al*[52] 2006	145	NAFLD and matched healthy controls	Biopsy	Severity of histological feature of NAFLD
Targher *et al*[55] 2006	200	Type2 diabetes +/- NAFLD	US	HOMA-IR
Aygun *et al*[49] 2008	80	NAFLD and matched healthy controls	US	NAFLD and BMI
Sookoian *et al*[7] 2008 (Meta-regression analysis)	3497	Subjects	US	ALT, GGT, NAFLD
Fracanzani *et al*[15] 2008	375	NAFLD and matched population controls	US and biopsy	Steatosis, age and blood pressure
Kim *et al*[42] 2009	1021	Cross sectional	US	NAFLD with MS
Petit *et al*[58] 2009	101	Type 2 diabetes	MRS	Age, no association with steatosis
Ramilli *et al*[43] 2009	154	Referred subjects for abdominal US	US	NAFLD
Wang *et al*[130] 2009	170	Healthy subjects	US	ALT levels
Salvi *et al*[50] 2010	220	NAFLD, healthy controls		NAFLD and MS
Mohammadi *et al*[44] 2011	335	Male NAFLD controls	US	NAFLD with or without MS
Neri *et al*[110] 2011	90	Chronic hemodialysis plus steatosis and healthy controls	US, biopsy	Histological steatosis
Valenti *et al*[131] 2011	506	NAFLD	US	Systolic blood pressure, glucose, LDL, abdominal circumference, age, ferritin
Huang *et al*[46] 2012	2590 + 6042	Chinese population +/- NAFLD	US	NAFLD
Kang *et al*[47] 2012	633	Non-diabetic +/- NAFLD	US	NAFLD with or without MS
Thakur *et al*[51] 2012	80	Non-diabetic NAFLD, healthy controls	US	NAFLD
Kim *et al*[132] 2013	769	Healthy subjects	US	Upper normal range of ALT in women with NAFLD
Oni *et al*[13] 2013 Systematic review	16 studies	Population, cross- sectional, case-control	US, biopsy	Positive association between IMT and NAFLD
Petta *et al*[126] 2013	162 + 267	NAFLD and validation cohort	Biopsy	PNPLA3 polymorphism in younger patients
Kim *et al*[133] 2014	4437	Type 2 diabetes	US	NAFDL with insulin resistance

US: Ultrasonography; MS: Metabolic syndrome; IMT: Intima-media thickness; NAFLD: Nonalcoholic fatty liver disease; MRS: Magnetic resonance spectroscopy.

availability within the liver and insulin resistance inhibit apolipoprotein B (APO-B) degradation with formation of more VLDL. The increased level of APO-B, a large protein involved in the transport of triglycerides and cholesterol from the liver to peripheral tissues, which is the primary protein component of the atherogenic LDL particles, is also responsible for accelerated atherosclerosis. The altered ratio between APO-B and apolipoprotein A-1 (APO-A1), the protein component of the anti-atherogenic HDL particles, has been suggested to be a good predictor of cardiovascular risk, even better than HDL/LDL ratio, with more cholesterol deposited in the arterial wall when the ratio is higher[140-142].

INFLAMMATION

Several results obtained in animal models have shown that lipid accumulation in the liver leads to sub-acute hepatic inflammation followed by cytokine production *via* the NF-κB pathway, in turn followed by increase in insulin resistance. Activation of the NF-kB pathway in the liver of patients with NASH leads to increased transcription of several pro-inflammatory gene that mediate the progression of systemic, low-grade inflammation. In line with these results a growing body of evidence indicates the atherosclerosis is proportional to the severity of liver damage, with CVD accelerated in patients with NASH but to a much less extent in those with simple steatosis[143]. Although the relation between hepatic inflammation and atherosclerosis is still matter of debate, these results are very suggestive for a role played by inflammation in atherosclerosis acceleration. In addition, the evidence that serum and liver tissue proinflammatory markers, such as C reactive protein, IL6, TNF and TGF-β and procoagulant factors, fibrinogen, and plasminogen activator inhibitors are increased in patients with accelerated atherosclerosis reinforces this hypothesis[11]. Furthermore, high-sensitivity C-reactive protein, biomarker of sub-clinical inflammation and of cardiovascular and cerebrovascular disease was found the strongest independent variable associated with NAFLD development in a case-control study[144].

OXIDATIVE STRESS

Several studies have shown that in patients with NAFLD, and in particular in those with NASH, there is evidence of systemic inflammation and increased oxidative stress, relevant factors contributing to metabolic alterations and cardiovascular damage. Altered oxidant/antioxidant status and subclinical inflammation are believed to play a key role in the pathogenesis of atherosclerosis. Hyper-

glycemia and inflammation increase the production of reactive oxygen species (ROS) triggering oxidative stress which is responsible for oxidation of carbohydrates, lipids and protein. Oxidation of polyunsaturated fatty acids gives raise to the production of malonylaldehyde (MDA), 4-hydroxy-nonenal (HNE) and 4-oxy-2-nonenal (ONE). Increases of these markers, have been detected in patients with NAFLD and correlated to vascular damage as well as in animal models and *in vitro* studies[145]. Furthermore, it has been found that specific localization in liver tissues cells of oxidized phosphatidylcholine (oxPC, a lipid peroxide that serves as a ligand for scavenger receptors), suggests that neutrophil myeloperoxidase-derived oxidative stress may be crucial in the progression of steatotic liver disease[146].

In addition inflammation and oxide-reductive imbalance may play an important role in the progression of NAFLD and atherosclerotic disease as documented in patients in chronic hemodialysis in which a progressive chronic liver and vascular damage developed faster than in patients without renal failure[110].

The "oxidative stress theory" implies that the initial phase of atherosclerosis is sustained by oxidation of LDL which accumulate in the arterial wall and are taken up by macrophages which, in turn, transform into foam cells. The increased serum levels of LDL and several other pathways of oxidative stress present in patients with NAFLD will accelerate atherosclerosis process[147]. Interestingly the "iron theory" of atherosclerosis, suggested by Sullivan[148], emphasizes the role of iron as a free radical inducer through oxidative stress could find consistency in patients with NAFLD. In fact the high prevalence of increased ferritin, detected in roughly 30% of the patients, could partly account for the accelerated atherosclerosis of these patients. Recent data point out that the relation between increased ferritin and early atherosclerosis, as evaluated by increased IMT, is valid only in patients negative for HFE mutations, suggesting that patients positive for these mutations with lower level of serum hepcidin may be protected from atherosclerosis[131]. This could explain the apparent paradox of the role of iron in atherosclerosis despite the lack of evidence of increased CVD in patients with hereditary hemochromatosis.

Thus, results from experimental and clinical studies suggest that oxidative stress is increased in patients with NAFLD/NASH and that adipose tissue is very likely the major source of ROS which in turn will cooperate in accelerating atherosclerosis.

ADIPOKYNES IMBALANCE

The increase in adipose tissue and chronic inflammation cause an imbalance in adipokines secretion, in particular a reduction of adiponectin and an increase in leptin, two adipokines with opposite function. Adiponectin has been shown to have anti-inflammatory and anti-fibrotic capability and it is believed that its low level may facilitate the progression of CVD and of steatosis to NASH, in addition has been reported to have specifically antiatherogenic properties, whereas leptin, found increased in NAFLD, has been shown in animal models to exert opposite effect. Furthermore, leptin has a multifunctional role in inflammation, that is fundamental in the atherogenic pathway, acting as a proinflammatory stimulus. Among other factors released within adipose tissue, TNF-α promotes lipolysis and increases FFAs; both TNF-α and IL-6 are related to mitochondrial and endothelial dysfunction, to atherosclerosis and cardiovascular disease; at the same time the reduction of protective adipokynes as adiponectin improves vascular damage[149-151].

COAGULATION IMBALANCE

Dyslipidemia, lipid-based oxidative injury and necroapoptosis underpin a multifactorial disorder which may result in a hypercoagulable state. This was suggested on the basis of the evidence of long-lasting ongoing inflammatory state associated with this condition and on epidemiological studies, although someone argues that despite an increased CVD risk, no strong evidence has been reported that patients with NAFLD are at increased risk of thrombosis.

Although a direct link between NAFLD and coagulation has not yet established, several cross-sectional studies indicate that NAFLD and NASH are associated with a systemic prothrombotic state possibly through the release of procoagulant factors from steatotic liver (C-reactive protein, plasminogen activator inhibitor-1, fibrinogen), in addition high levels of factors VIII, IX, XII, VII, von Willebrand, soluble tissue factor, platelet hyperaggregability, procoagulant microparticles are found in NAFLD independently of age, BMI waist circumference, plasma triglycerides and insulin resistance[152-155] as well as in cirrhosis[156]. A possible explanation for the failure to ascertain a causal effect between NAFLD and an impairment of coagulation is the lack of appropriate tests. In fact, coagulation evaluated by traditional global tests such as the prothrombin and activated partial thromboplastin times (PT/APTT) or through the measurement of the individual pro- or anti-coagulants does not allow to detect the complex interplay between the procoagulants (*i.e.*, thrombin-generation drivers) and their naturally-occurring anticoagulant counterparts (*i.e.*, thrombin-neutralization drivers) operating *in vivo*. However despite the lack of conclusive data, prelimary results seem to suggest the existence a coagulation unbalance between production and/or half-life of both pro- and anticoagulant proteins[157] as shown in Figure 3.

INCREASED ASSUMPTION OF FRUCTOSE

Patients with NAFLD frequently consume a large amount of fructose, a highly lipogenic sugar which is a common component in all major caloric sweeteners (sucrose, high-fructose corn syrup, honey and fruit juice concentrates) and soft drinks[128,158]. Fructose consump-

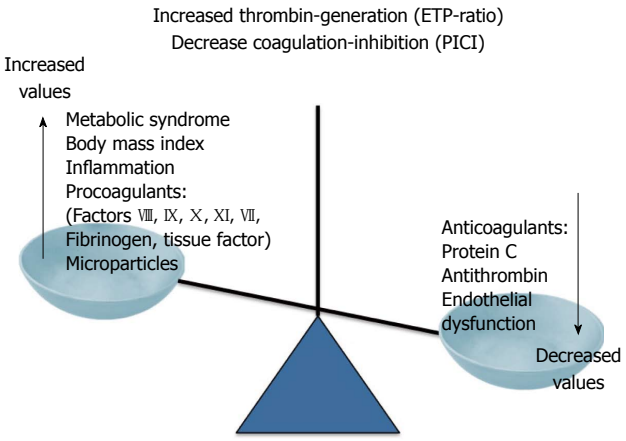

Figure 3 Causes and consequences of the procoagulant imbalance in patients with nonalcoholic fatty liver disease. ETP-ratio: Ratio of the endogenous thrombin potential measured in the presence or absence of thrombomodulin; PICI: Protac®-induced coagulation inhibition.

tion promotes hepatic *de novo* lipogenesis and intrahepatic lipids deposition, inhibition of mitochondrial beta-oxidation of long-chain fatty acids , triglycerides formation and steatosis, increased plasma levels of fasting small dense LDL-C, oxidized LDL-C, and activation of inflammatory pathways. All these fructose-induced effects synergize with the metabolic alterations typical of NAFLD playing a key role in atherosclerosis acceleration. This mechanism is particularly important in lean subjects who very often consume a large amount of fructose.

CONCLUSION

All data reported in this review provide evidence of a strong association between NAFLD and subclinical atherosclerosis. Furthermore, current evidence suggests that NAFLD, although considered the hepatic manifestation of the metabolic syndrome, is emerging as an independent risk factor for the occurrence and progression of CVD. The findings and conclusions are similar in retrospective and prospective studies, which evaluated different manifestations of subclinical atherosclerosis (increased IMT, endothelial dysfunction, arterial stiffness, impaired left ventricular function and coronary calcification).

A general agreement emerging from these studies indicates that patients with NASH are at higher risk of CVD than those with simple steatosis, emphasizing the role of chronic inflammation in the pathogenesis of atherosclerosis of these patients.

It is very likely that the different mechanisms involved in the pathogenesis of atherosclerosis in patients with NAFLD have a different relevance in the different patients according to individual genetic background.

In conclusion, in the presence of NAFLD greater emphasis should be placed on the cardiovascular risk with patients undergoing a complete cardiovascular evaluation to prevent future atherosclerotic complications. Specific life-style modification and aggressive pharmaceutical modification will not only reduce the progression of liver disease, but also reduce morbidity for cardiovascular disease improving over-all prognosis and survival.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Biomarkers for pancreatic cancer: Recent achievements in proteomics and genomics through classical and multivariate statistical methods

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Abstract

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases. A valid alternative to the usual invasive diagnostic tools would certainly be the determination of biomarkers in peripheral fluids to provide less invasive tools for early diagnosis. Nowadays, biomarkers are generally investigated mainly in peripheral blood and tissues through high-throughput omics techniques comparing control *vs* pathological samples. The results can be evaluated by two main strategies: (1) classical methods in which the identification of significant biomarkers is accomplished by monovariate statistical tests where each biomarker is considered as independent from the others; and (2) multivariate methods, taking into consideration the correlations existing among the biomarkers themselves. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and prognostic performances which are superior to single markers in terms of sensitivity, specificity and robustness. Multivariate techniques are usually applied with variable selection procedures to provide a restricted set of biomarkers with the best predictive ability; however, standard selection methods are usually aimed at the identification of the smallest set of variables with the best predictive ability and exhaustivity is usually neglected. The exhaustive search for biomarkers is instead an important alternative to standard variable selection since it can provide information about the etiology of the pathology by producing a comprehensive set of markers. In this review, the most recent applications of the omics techniques (proteomics, genomics and metabolomics) to the identification of exploratory biomarkers for PC will be presented with particular regard to the statistical methods adopted for their identification. The basic theory related to classical and multivariate methods for identification of biomarkers is presented and then, the most recent applications in this field are discussed.

Key words: Pancreatic cancer; Biomarker identification; Multivariate analysis; Principal component analysis; Ranking principal component analysis

Core tip: Biomarkers are statistically identified as significant by: (1) classical statistical tests where each biomarker is independent from the others; and (2) multivariate methods that take into consideration the correlation among the biomarkers. This last approach provides pools of biomarkers with superior diagnostic and prognostic performances. Multivariate techniques are often applied with variable selection procedures to provide the smallest set of biomarkers with the best predictive ability. The exhaustive identification is instead a valid alternative since it can provide comprehensive information about the etiology of the pathology. The most recent applications of the omics approaches to the identification of biomarkers for PC are presented, with particular regard to the statistical methods adopted.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases; its early detection is therefore fundamental since surgery at an early disease stage is the preferred and most promising therapy. About 20% of patients can be operated on at time of diagnosis; the 5-year survival rate for not-operable patients is about 1%, while the 5-year survival after surgery is about 20% without an adjuvant therapy and about 25%-30% with the therapy[1-3]. The lack of early symptoms and the high aggressiveness are the main causes of late diagnosis and high mortality of this disease. Therefore, the search for biomarkers of early diagnosis is highly recommended to improve the early diagnostic rate, thus improving patients' prognosis.

Diagnosis is usually based on invasive techniques [ultrasound endoscopy (EUS), explorative laparoscopy or laparotomy] or on methods that can be at least inconvenient for patients [computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde or magnetic resonance cholangiopancreatography (ERCP and MRCP)][2].

A valid alternative would certainly be the determination of specific biomarkers in peripheral fluids in order to provide less invasive tools for early diagnosis. In this direction, the most recent efforts in the field of biomarker identification for PC are directed. A wide range of serum markers for PC has been reported[2,4] but few of them are exploited in clinical routine since they show low sensitivity and/or specificity in general. Bünger *et al*[2] reviewed about 43 serum biomarkers for PC divided into four main groups: carbohydrates (CA19-9, CA 50, CA 125, CA195, CA 72-4), carcinoembryonic antigens, other markers and the combination of different markers.

Together with diagnostic markers, great efforts have recently been made to identify predictive and prognostic biomarkers for PC. Tissue biomarkers for the prognosis of pancreatic ductal adenocarcinoma (PDAC) have recently been reviewed by Jamieson *et al*[4]. The considerations that drive the search for diagnostic biomarkers also apply for predictive and prognostic ones and the identification of markers from peripheral blood should be the best alternative to provide prognostic methods that are less invasive for patients. Nowadays, both diagnostic and prognostic/predictive biomarkers are generally investigated mainly in peripheral blood or tissues through high-throughput omics techniques. The results can be evaluated by two different strategies, namely by: (1) classical statistical methods consisting of the use/identification of significant biomarkers by monovariate statistical tests where each biomarker is considered as independent from the others; and (2) multivariate methods, able to take into consideration the multivariate structure of the data and the correlations among the potential biomarkers. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and/or prognostic performance superior to single markers in terms of sensitivity, specificity and robustness.

It is important to point out that biostatistical methods can usually be defined as multivariate (several endpoints and several predictors) or multivariable (one endpoint, several predictors). In this specific context, the authors will generally apply the term multivariate to identify methods that allow the evaluation of the correlations between the variables, *i.e.*, their synergisms and/or antagonisms.

Multivariate techniques are usually applied with variable selection procedures[5,6] to provide a set of candidate biomarkers with the best predictive ability; however, standard selection tools are aimed at the identification of the smallest set of variables with the best predictive ability. It is the authors' opinion that exhaustivity should also be addressed[7]. Biomarkers are useful not only for diagnostic/prognostic purposes but also to better understand the etiology of pancreatic cancer. From this point of view, the exploitation of high-throughput methods provides a lot of information that should not be neglected. The exhaustive identification of all possible biomarkers showing large correlations could provide information about the overall mechanism of action of the disease, thus opening the way towards new therapeutic strategies.

In this review, the most recent applications of the omics approaches (proteomics, genomics and metabolomics) for the identification of biomarkers for pancreatic cancer will be presented, with particular regard for the statistical methods adopted for their identification, focusing especially on exploratory biomarkers. High-throughput techniques will probably be the future in the field of searching for exploratory biomarkers due to the great amount of information they convey. Moreover, the possibility of combining the results emerging from proteomic, genomic and metabolomic studies with clinical information can provide exhaustive panels of markers, thus improving their predictive performance with better sensitivity and specificity.

First, the theory of the classical and multivariate methods for identification of biomarkers will be presented, followed by the most recent applications in this field.

STATISTICAL METHODS

The statistical methods presented here can be divided into four main groups: (1) classical methods for identification of biomarkers based on monovariate approaches; (2) tools for biomarkers search based on multivariate approaches; (3) methods for the analysis of survival outcomes; and (4) other methods. Only the tools recently applied to the specific case of pancreatic cancer will be

Table 1 Statistical methods adopted in the identification of biomarkers for pancreatic cancer

Type of statistical method	Method adopted
Classical mono- and multi-variate methods	Student *t*-test (parametric)
	Mann-Whitney *U*-test (non-parametric)
	T^2 Hotelling
	ANOVA and MANOVA
	Bayes factors
Unsupervised pattern recognition methods	Principal Component Analysis
	Cluster Analysis
	Multidimensional Scaling
Supervised classification methods	SIMCA
	Ranking-PCA
	O-PLS
	CART
	Random Forests
Methods for determining survival outcomes	Kaplan Meyer functions
	Cox Regression
Other methods	PAM
	Metropolis algorithm and Monte Carlo simulation

PCA: Principal component analysis; SIMCA: Soft independent model of class analogy; PLS: Partial least squares; CART: Classification and regression tree.

briefly presented as an exhaustive treatment of all multivariate procedures in the field of searching for biomarkers is out of the scope of the present review.

CLASSICAL MONOVARIATE METHODS FOR IDENTIFICATION OF BIOMARKERS

The classical approach to the identification of markers of a specific disease is the evaluation of which variables show a different behavior between two groups of samples (control *vs* pathological, control *vs* drug-treated, *etc*.). The easiest statistical way to solve this problem is the application of classical statistical tests to each biomarker candidate separately and the calculation of the type I error that can be accomplished comparisonwise (for each hypothesis independently) or experimentwise (testing all hypotheses together). The second alternative is preferred since the type I error probability increases as the number of tests increases. The identification of significant markers is therefore accomplished by the Student's *t*-test for each variable independently and by applying a correction taking into account the number of multiple tests available: Bonferroni's method with subsequent modifications[8] or the corrections proposed by Dunn and Sikak and by Dunnet[9-11]. This approach is incorrectly defined as multivariate since it does not take into consideration the correlations eventually existing between the variables. The same approach can also be applied to non-parametric tests to be exploited when the number of samples is too small or when the assumptions at the basis of parametric tests are not verified. Among them, the most widespread in the biomedical field is the Mann-Whitney *U*-test[8].

An alternative is the exploitation of global tests aimed at demonstrating a global hypothesis (*e.g*., the effect of a therapy) considering all the tests simultaneously; an example is the Hotelling's T^2 test[8].

Classical procedures also comprise the approach based on the analysis of variance both in its two-way (ANOVA) or multi-way (MANOVA) versions[8]. In this case, it is also possible to compare more than two groups of samples.

An alternative to classical hypothesis testing is the use of the Bayes factors[12], providing a more robust approach. For comparing two hypotheses H_1 and H_2, this factor may be approximated as the ratio of the marginal likelihood of the data under the two hypotheses and can be interpreted as follows: $B \leqslant 0.1$: strongly against H_1; $0.1 < B \leqslant 1$: against H_1; $1 \leqslant B < 3$: barely worth mentioning for H_1; $3 \leqslant B << 10$: substantially for H_1; $B > 10$: strongly for H_1.

MULTIVARIATE METHODS FOR IDENTIFICATION OF BIOMARKERS

Methods that can be properly defined multivariate are based on the comparison of two or more groups of samples taking into account the relationships between the variables, rather than considering them as independent. This approach is certainly more effective since it is fundamental to consider the synergic or antagonistic effects of different factors, *i.e*., their interactions. Moreover, when independent tests are performed on several factors and a high correlation exists between them, the outcome of the test can be completely wrong[13]. The methods here presented belong to two approaches: (1) unsupervised methods (pattern recognition methods), in which no a priori information is assumed and the evaluation of the existence of groups of samples is suggested by the statistical method itself; and (2) supervised methods (classification tools), in which the a priori information is provided in terms of membership of each sample to a specific class: the statistical method is therefore aimed at the identification of the variables responsible for the separation of the samples in the different classes.

Here, only the methods most recently applied in the literature to the case of pancreatic cancer will be briefly discussed. The methods presented are listed in Table 1.

UNSUPERVISED AND PATTERN RECOGNITION METHODS

Principal component analysis

Principal component analysis (PCA)[14,15] represents the objects in a new reference system characterized by new variables called principal components. The first principal component accounts for the maximum variance contained in the original dataset, while subsequent components account for the maximum residual variance. They are calculated hierarchically, so that systematic variations (*i.e*., information) are explained in the first components while experimental noise and random variations are contained in the last ones. The components are linear combinations of the original variables and are orthogo-

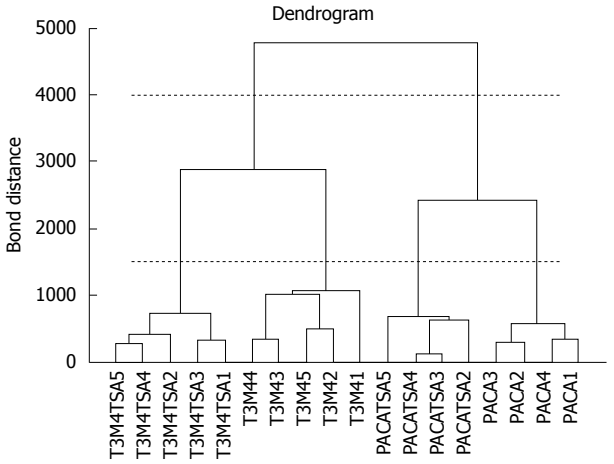

Figure 1 Example of a dendrogram built using Ward's linkage method and euclidean distances. Data refer to samples from two pancreatic cancer cell lines treated or not with trichostatin A.

nal to each other, thus containing independent sources of information. They are often used for dimensionality reduction by considering a smaller number of significant components containing only relevant information.

The graphical representation of the scores (the coordinates of the samples in the new reference system) in the space of the principal components allows the identification of groups of samples showing a similar behavior (samples close one to the other in the graph) or different characteristics (samples far from each other). The corresponding loading plot (representing the loadings, *i.e.*, the coefficients of the linear combination describing each principal component) identifies the variables that are responsible for the analogies or the differences detected for the samples in the score plot.

Cluster analysis

Cluster analysis techniques[14-16] allow the identification of groups of samples or of variables in a dataset by investigating the relationships between the samples or the descriptors. Agglomerative hierarchical methods[14,15] are the most widespread, grouping the samples on the basis of their similarity. The most similar samples or groups are linked first. The final result is a graph, called a dendrogram, where the samples are represented on the X axis and are connected at decreasing levels of similarity along the Y axis. The groups can be identified by applying a horizontal cut of the dendrogram and identifying the number of vertical lines crossed by the horizontal cut. Figure 1 reports a dendrogram where cutting at level 4000 produces only two clusters (the 2 different tumor cell lines), while cutting at level 1500 produces 4 clusters (PACA and T2M4 cell lines, treated and untreated with trichostatin A). The results of hierarchical clustering strongly depend on the measure of similarity and on the linking method adopted. Clustering techniques can be applied to the original variables or to the relevant principal components[16].

Multidimensional scaling

Multidimensional scaling (MDS)[17,18] is aimed at dimensionality reduction and graphical representation of the data. Given a set of *n* objects and a measure of their similarity, MDS searches for a low dimensional space in which the objects are represented by points in the space so that the distances between the points match as much as possible with their original similarities[17]. There are several different approaches to MDS depending on the measure of the similarities matching, on the metrics, on the method used to compute the similarities and on the way the samples configuration is obtained[19,20]. Shepard[21,22] and Kruskal[23] provided an extension of classical MDS to the study of nonparametric similarities.

SUPERVISED CLASSIFICATION METHODS

Classification tools are supervised methods able to separate the objects in the classes present (known a priori, *e.g.*, control *vs* pathological) and provide the variables most responsible for their belonging to different classes (candidate biomarkers). The final aim of their application in the biomedical field is both the development of diagnostic tools and the identification of the differences existing between the classes to shed light on the etiology of a disease or the effect of a new drug. Here, only the methods already applied to the identification of biomarkers for PC will be described.

Soft independent model of class analogy

The soft independent model of class analogy (SIMCA) method[24-27] is based on the independent modelling of each class by means of PCA. Each class is described by its relevant principal components. The samples belonging to each class are contained in the so-called SIMCA boxes, defined by the relevant components of each class. The classification of each sample with SIMCA is not affected by experimental uncertainty and random variations since each class is modelled only by its relevant components. This method is also useful when more variables than objects are available since it performs a substantial dimensionality reduction.

The identification of the candidate biomarkers by SIMCA can be accomplished by the analysis of the discrimination power (DP), a measure of the ability of each variable to discriminate between two classes at a time. The greater the DP, the more a variable weighs on the classification of an object in one of the two classes compared.

Ranking PCA

Ranking PCA is a ranking method proposed by Marengo *et al*[7], Robotti *et al*[28] and successively applied by Polati *et al*[29], based on the description of the original data by means of principal components. The use of PCA in the field of identification of biomarkers in the omics sciences is particularly effective since it allows the relationships be-

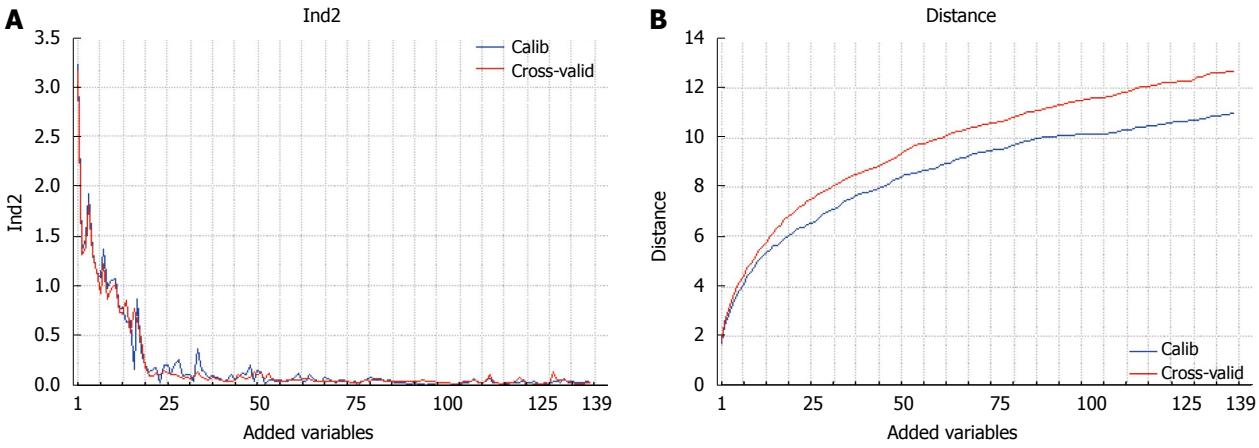

Figure 2 Example of the graphical representation of the results from Ranking-PCA. Trend of Ind_2 *vs* the increasing number of variables added to the model (A); trend of the distance between the two class centroids *vs* the number of variables added to the model (B). Variables on the X-axis are reported in the order in which they are included in the model. Both calibration and cross-validation results are reported.

tween the variables to be taken into consideration, providing sets of correlated biomarkers with a similar function and the possibility of solving problems where the number of variables is larger than the number of samples.

PCA is used here to describe the data coupled to a ranking procedure of the candidate biomarkers in a forward search: one variable is added at each cycle. The first variable selected is the one providing the best separation between the classes on the first principal component. The addition of another discriminating variable further improves the distance between the two classes on the first principal component. If a non-discriminating variable is successively added, the two classes will not be further separated on the same component. Sometimes, more than one component could be necessary for class separation: in this case, different independent sources of information related to the class structure are present in the data and the subsequent principal component accounting for class separation will be included in the model.

The proposed method allows the ranking of the variables according to their discrimination ability, thus assuring the exhaustiveness of the results. The result can be presented in graphical form (Figure 2), where the classification performance or the class distance are reported as a function of the variables added to the model.

Orthogonal partial least squares

Partial least squares (PLS)[14,15,30] establishes a relationship between one or more dependent variables (Y) and a group of descriptors (X). X and Y variables are modeled simultaneously to find the latent variables (LVs) in X that will predict the LVs in Y. These LVs are calculated hierarchically, as for PCA. PLS was originally set up to model continuous responses but it can be applied even for classification purposes (PLS-DA) by establishing an appropriate Y related to the association of each sample to a class. The regression is then carried out between X-block variables and the Y just established. Orthogonal PLS (OPLS)[31] is a modification of PLS developed for highly decorrelated datasets.

Classification and regression tree

Classification trees[24] are built by subsequent divisions (splits) of subgroups of the original data D in two descending subgroups with the aim of classifying the data in homogeneous groups as much as possible, one different from the others. It is possible to derive a tree diagram where, starting from the root node (where the data D are not separated), a series of nodes and branches separate; each node h represents a subgroup of D. Nodes not undergoing a further split are called terminal nodes: a mode for y is associated with each terminal node.

Starting from the root node h_1, data are separated in a series of splits: in each node, the split giving the most homogeneous division of the data in the two descendent nodes is selected.

Random forests

Random forests[32] is an extension of classification trees and is structured to grow many classification trees. To classify a new object, the new object is first classified by each independent tree in the forest. The forest chooses the most recurrent classification (over all the trees in the forest).

The error rate depends on the correlation between any two trees in the forest (increasing the correlation increases the forest error rate) and the strength of each individual tree in the forest (inversely correlated to the tree error rate).

METHODS FOR THE ANALYSIS OF SURVIVAL OUTCOMES

In clinical trials it is usually important to evaluate the time until the participants present with a particular event (endpoint), *i.e.*, a clinical outcome (death, recurrence of a disease, remission *etc.*). All participants are followed from a certain starting point (operation, starting of a therapy, di-

agnosis, *etc*.) up to the moment when the event occurs (the time requested is recorded). However, often the outcome of some participants is unknown: when the study ends before all participants have presented the event or when some participants withdraw from the study. In these cases (censored data), the time of follow-up is recorded.

Kaplan-Meier estimates of survival functions

The Kaplan-Meier method[33] is used to provide survival functions and can also be effectively applied to censored data. It provides a survival curve where time is reported on the X-axis, while the cumulative survival probability is on the Y-axis. The time corresponding to the point where the curve crosses 50% survival is the estimate of median survival. Kaplan-Meier curves can be compared across groups by mainly applying two non-parametric tests (the log rank test[34] and the generalized Wilcoxon test[35]). The generalized Wilcoxon test[35] is a weighted alternative where early time points weigh more than late ones; this is preferred when the effect of an experimental condition vanishes with time. The log rank test instead is preferred to detect differences during all of the follow-up period.

Cox regression

Cox regression[36] is applied to evaluate the effect of several risk factors on survival, defining the hazard as the probability of the endpoint. The hazard is modeled as:

$$ln\,[H(t)/H_0(t)] = b_1X_1 + b_2X_2 + \cdots + b_kX_k$$

where $H(t)/H_0(t)$ is the so-called hazard ratio (HR), X_1 ... X_k are predictor variables and $H_0(t)$ is the baseline hazard at time. In general, the HR may assume only positive values: if it equals 1, the groups show a not statistically different survival; if it is smaller than 1, a subject with a higher value for X has lower risk than a subject with a lower value for X; the opposite behavior is obtained if the HR estimate is larger than 1.

The Cox model works under the so-called proportional hazards assumption: the ratio between the hazards of two patient groups remains constant over the complete follow-up period. Since a HR is calculated in Cox regression, this estimate should apply to all death times: this simplification is only justified if the group difference remains constant over the whole range of follow-up time.

If the monovariate Cox regression is extended to include more than one X variable (multivariate Cox regression), the effect of the interaction between different factors can be evaluated.

OTHER METHODS

Prediction analysis for microarrays

Prediction analysis for microarrays (PAM) performs sample classification from gene expression data and survival outcomes, exploiting the nearest shrunken centroid approach[37]. A standardized centroid for each class is computed. The nearest centroid classification compares the gene expression profile of a new sample to each class centroid: the class whose centroid is closest in squared distance is the predicted class for the new sample.

Nearest shrunken centroid classification[37] is a modification of this approach. When PAM is applied to survival outcomes, supervised principal components analysis[38] is performed, where, instead of using all the genes to perform PCA, only a subset of genes is used, *i.e*., those highly correlated with survival.

Metropolis algorithm and Monte Carlo simulation

Monte Carlo methods[39] are computational algorithms that rely on repeated random sampling to obtain numerical results; simulations are run several times to calculate probabilities. They are useful for simulating systems with several degrees of freedom and modeling systems characterized by large uncertainty in inputs.

The Metropolis algorithm[40] is used to generate a series of numbers, X_1, X_2, .., X_n with a distribution fixed a priori. The method is based on the generation of numbers that are accepted or rejected to obtain the selected type of distribution.

The Metropolis algorithm can be implemented in Monte Carlo simulations to perform random sampling. The Monte Carlo optimization can be used in biomarkers search to determine the coefficients of model containing the relevant biomarkers.

STUDIES FOR THE IDENTIFICATION OF BIOMARKERS

Studies based on serum and tissue biomarkers determined by non-omics techniques

The studies based on serum and tissue biomarkers determined by non-omics techniques usually exploit monovariate and multivariate Cox regression to evaluate the effect played by different factors on time to progression (TTP) and overall survival (OS). They will be presented here, divided into prognostic/predictive biomarkers and diagnostic biomarkers.

Prognostic and predictive biomarkers

Recent studies about prognostic and/or predictive biomarkers (Table 2) determined in serum or plasma regard the determination of glycoproteins, both alone or associated with other markers, and the determination of circulating factors of the insulin-like growth factor. Three recent studies have been published based on CA19-9, one of the most debated biomarkers for PC. The first study, by Boeck *et al*[41], includes 115 patients with histologically confirmed advanced PC treated with first-line therapy. The novelty of this study is the modelling of the effect of CA 19-9 kinetics by treating it as a time-varying covariate. For CA 19-9 kinetics during chemotherapy, data from 69 patients (TTP) and 84 patients (OS) were available. The proposed approach allowed the modeling of the effect of log (CA 19-9) measured during therapy on

Table 2 Studies based on serum and tissue biomarkers through non-omics techniques

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
41	P	CA 19-9	S	Pretreatment CA 19-9: 115 patients from 5 German centers; 73% treated within prospective clinical trials. Median TTP: 4.4 mo; median OS: 9.4 mo. CA 19-9 kinetics during chemotherapy: 69 patients (TTP) and 84 patients (OS)	Elecsys assay	Cox proportional hazards regression; for CA 19-9 kinetics, CA 19-9 was treated as a time-varying covariate	Univariate analysis: log (CA 19-9) associated with TTP (HR = 1.24; $P < 0.001$) and OS (HR = 1.16; $P = 0.002$). Multivariate analysis: results confirmed. Log(CA 19-9) kinetics during chemotherapy: significant predictor for TTP in univariate analyses (HR = 1.48; $P < 0.001$) and multivariate (HR = 1.45; $P < 0.001$) and for OS (univariate: HR = 1.34; $P < 0.001$; multivariate: HR =1.38; $P < 0.001$)
42	P	CA 19-9, CEA, CRP, LDH and bilirubin		291 patients; 253 patients (87 %) received treatment within prospective clinical trials. Median TTP: 5.1 mo. Median OS 9.0 mo	Elecsys assay	Kaplan Meier method and Cox proportional hazards regression	Univariate analysis: pre-treatment CA 19-9 (HR = 1.55), LDH (HR = 2.04) and CEA (HR = 1.89) significantly associated with TTP. Baseline CA 19-9 (HR = 1.46), LDH (HR = 2.07), CRP (HR = 1.69) and bilirubin (HR = 1.62) significant prognostic factors for OS. Multivariate analyses: pre-treatment log (CA 19-9) for TTP and log (bilirubin) and log (CRP) for OS had an independent prognostic value
44	P	IGFs	S and P	80 patients received treatment (40 Ganitumab; 40 placebo)	Immunoassays	Kaplan Meier method and Cox proportional hazards regression	Ganitumab associated with improved OS *vs* placebo (HR = 0.49; 95%CI: 0.28-0.87)
45	P	TROP2	T	197 patients; subgroup of 134 patients treated surgically	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	TROP2 overexpression observed in 109 (55%) patients and associated with decreased OS ($P < 0.01$). Univariate Analysis: TROP2 overexpression correlates with lymph node metastasis ($P < 0.04$) and tumor grade ($P < 0.01$). In the subgroup of patients treated surgically, TROP2 overexpression correlated with poor progression-free survival ($P < 0.01$). Multivariate analyses: TROP2 is an independent prognosticator
46	P	JAM-A	T	186 patients; subgroup of 83 patients treated surgically	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	Low expression of JAM-A observed in 79 (42 %) patients and associated with poor OS ($P < 0.01$). Univariate analysis: low expression of JAM-A correlates with positive lymph node status ($P = 0.02$), the presence of distant metastasis ($P = 0.05$), and tumor grade ($P = 0.04$). In the subgroup of patients with surgically resected PC, low expression of JAM-A correlated with decreased progression-free survival ($P < 0.01$). Multivariate analysis: JAM-A was an independent predictor of poor outcome
47	P	TBX4	T	77 stage Ⅱ PDAC tumors	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	48 cases (62.3%) expressed TBX4 at a high level. No significant correlation between TBX4 expression and other clinicopathological parameters, except tumor grade and liver metastasis recurrence. Survival of patients with TBX4-high expression significantly longer than those with TBX4-low expression ($P = 0.010$). Multivariate analysis: low TBX4 expression independent prognostic factor for OS. TBX4 promoter methylation status frequently observed in PDAC and normal adjacent pancreas
48	P	HSP27	T	86 patients	Tissue microarray (TMA) analysis	Kaplan Meier method and Cox proportional hazards regression	HSP27 expression found in 49% of tumor samples. Univariate analyses: significant correlation between HSP27 expression and survival. Multivariate Cox-regression: HSP27 expression emerged as an independent prognostic factor. HSP27 expression also correlated inversely with nuclear p53 accumulation
49	P	dCK	T	45 patients with resected PDAC received adjuvant gemcitabine based-therapy in multicenter phase 2 studies	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	Median follow-up: 19.95 mo (95%CI: 3.3-107.4 mo). Lymph node (LN) ratio and dCK protein expression significant predictors of DFS and OS in univariate analysis. Multivariate analysis: dCK protein expression the only independent prognostic variable (DFS: HR = 3.48, 95%CI: 1.66-7.31, $P < 0.001$, OS: HR = 3.2, 95%CI: 1.44-7.13, $P < 0.004$)

50	P	Notch3 and Hey-1	T	42 patients who underwent resection and 50 patients diagnosed with unresectable PDAC	Immunohistochemistry	Mann-Whitney U test, Wilcoxon test, Cox regression analysis, Kaplan-Meier analysis	All 3 Notch family members significantly elevated in tumor tissue. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1, and HEY-1 (all $P < 0.001$) in locally advanced and metastatic tumors compared to resectable cancers. In survival analyses, nuclear Notch3 and HEY-1 expression significantly associated with reduced OS and DFS following tumor resection with curative intent
51	D and P	21 biomarkers	P	clinically defined cohort of 52 locally advanced (Stage Ⅱ/Ⅲ) PDAC cases and 43 age-matched controls	Proximity ligation assay	Combination of the PAM algorithm and logistic regression modeling. Biomarkers that were significantly prognostic for survival were determined using univariate and multivariate Cox survival models	CA19-9, OPN and CHI3L1 were found to have superior sensitivity for pancreatic cancer *vs* CA19-9 alone (93% *vs* 80%). CEA and CA125 have prognostic significance for survival ($P < 0.003$)
52	D	83 circulating proteins	S	333 PDAC patients; 144 controls (benign pancreatic conditions); 227 healthy controls. Samples from each group split randomly into training and blinded validation sets. Panels evaluated in validation set and in patients diagnosed with colon (33), lung (62) and breast (108) cancers	bead-based xMAP immunoassays	A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set	Training set (160 PDAC, 74 Benign, 107 Healthy): panel of CA19–9, ICAM-1, and OPG discriminated PDAC from Healthy controls (SN/SP 88/90%), panel of CA 19–9, CEA, and TIMP-1 discriminated PDAC patients from Benign subjects (SN/SP = 76%/90%). Independent validation set (173 PDAC, 70 Benign, 120 Healthy): panel of CA 19–9, ICAM-1 and OPG demonstrated SN/SP of 78%/94%; panel of CA19–9, CEA, and TIMP-1 demonstrated SN/SP of 71%/89%. The CA19–9, ICAM-1, OPG panel is selective for PDAC and does not recognize breast (SP = 100%), lung (SP = 97%), or colon (SP = 97%) cancer
53	D and P	YKL-40, IL-6, and CA 19.9	P	559 patients with PC from prospective biomarker studies from Denmark ($n = 448$) and Germany ($n = 111$)	ELISA and chemiluminescent immunometric assay	Kaplan Meier method and Cox proportional hazards regression	Odds ratios (ORs) for prediction of PC significant for all biomarkers, with CA 19.9 having the highest AUC (CA 19.9: OR = 2.28, 95%CI: 1.97-2.68, $P = 0.0001$, AUC = 0.94; YKL-40: OR = 4.50, 3.99-5.08, $P = 0.0001$, AUC = 0.87; IL-6: OR = 3.68, 3.08-4.44, $P = 0.0001$, AUC = 0.87). Multivariate Cox analysis: high preoperative IL-6 and CA 19.9 independently associated with short OS (CA 19.9: HR = 2.51, 1.22–5.15, $P = 0.013$; IL-6: HR = 2.03, 1.11-3.70, $P = 0.021$). Multivariate Cox analysis of non-operable patients: high pre-treatment levels of each biomarker independently associated with short OS (YKL-40: HR = 1.30, 1.03-1.64, $P = 0.029$; IL-6: HR = 1.71, 1.33-2.20, $P = 0.0001$; CA 19.9: HR = 1.54, 1.06-2.24, $P = 0.022$). Patients with preoperative elevation of IL-6 and CA 19.9 had shorter OS ($P = 0.005$) compared to patients with normal levels (45% *vs* 92% alive after 12 mo)

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample: S: Serum; P: Plasma; T: Tissue; TTP: Time to progression.

the event (TTP or OS) rather than modeling the effect of the pretreatment value of log (CA 19-9).

In the second study, Haas *et al*[42] pooled pre-treatment data on CA 19-9, carcinoembryonic antigen (CEA), C-reactive protein (CRP), lactate dehydrogenase (LDH) and bilirubin from two multicenter randomized phase II trials and prospective patient data. Marker levels were assessed before the start of palliative first-line therapy for advanced PC and during treatment (for CA 19-9 only).

In the third study, Boeck *et al*[43] evaluated pre-treatment (palliative first-line chemotherapy) values and weekly values of cytokeratin 19-fragments (CYFRA 21-1), CA 19-9 and CEA in blood samples from patients with PC. CYFRA 21-1 are biomarkers for different epithelial diseases but their role in PC has not been investigated yet.

In Boeck *et al*[41] pre-treatment log (CA19-9) proved to be significantly associated with TTP and OS. Moreover, log (CA 19-9) kinetics after the start of treatment was found to be a significant predictor for both TTP and OS. Similar results were found by Haas *et al*[42] where pre-treatment CA 19-9, LDH and CEA levels were significantly associated

with TTP. Regarding OS, baseline CA 19-9, LDH, CRP and bilirubin were significant. Boeck *et al*[43] found that CYFRA 21-1 and CA 19-9 showed a high correlation with TTP and OS, while in multivariate analysis, only CYFRA 21-1 and performance status were independent predictors for OS.

McCaffery *et al*[44] assessed the predictive nature of baseline circulating factors of the insulin-like growth factor (IGF) axis on the treatment effect of ganitumab plus gemcitabine in metastatic PDAC. Baseline levels of IGFs/IGF binding proteins were analyzed in serum or plasma while mutations and gene expression were analyzed in archival samples. Ganitumab was associated with improved OS *vs* placebo. The treatment effect on improved OS was larger in patients with higher levels of IGF-1, IGF-2 or IGFBP-3, or lower levels of IGFBP-2. Interaction between treatment and IGFs/IGFBPs showed predictive potential for IGF-2 and IGFBP-2.

The studies about prognostic and/or predictive biomarkers determined in tissue samples, usually by immunohistochemistry or tissue microarray analysis (TMA), instead include the determination of glycoproteins, other proteins and enzymes.

Fong *et al*[45] investigated the expression of TROP2 (human trophoblast cell-surface) antigen, a glycoprotein found to be strongly expressed in a variety of human epithelial cancers, and the expression of junctional adhesion molecule A (JAM-A) antigen[46], a type I transmembrane glycoprotein, which has been recently shown to affect the prognosis of several malignancies. The two studies involved 197 and 186 patients with PDAC respectively. TROP2 overexpression was observed in 55% of patients, while low expression of JAM-A was observed in 42% of samples; both markers were significantly associated with decreased OS. They were both correlated with lymph node metastasis and tumor grade. In the subgroup of patients surgically treated with curative intent, TROP2 and low expression of JAM-A correlated with poor progression-free survival.

In the study by Zong *et al*[47], the expression of the T-box transcription factor 4 (TBX4) was investigated in 77 stage II PDAC tumors. 62.3% of cases expressed TBX4 at a high level. Significant correlation was only detected between TBX4 expression and tumor grade and liver metastasis recurrence. The survival with TBX4-high expression was significantly longer.

Applying tissue microarray (TMA) analysis, Schäfer *et al*[48] correlated heat shock protein 27 (HSP27) expression status with clinicopathological parameters in PDAC from 86 patients. HSP27 expression was found in 49% of tumor samples. A significant correlation was found with OS. The authors also assessed the impact of HSP27 on chemo- and radio-sensitivity directly in PC cells. HSP27 expression emerged as an independent prognostic factor and correlated inversely with nuclear p53 accumulation, indicating protein interactions between HSP27 and p53 or TP53 mutation-dependent HSP27-regulation. HSP27 overexpression rendered HSP27 low-expressing PL5 PC cells more susceptible to treatment with gemcitabine, while HSP27 protein depletion in HSP27 high-expressing AsPC-1 cells caused increased gemcitabine resistance.

Maréchal *et al*[49] identified deoxycytidine kinase (dCK), a recombinant enzyme, to be associated with prolonged survival after adjuvant gemcitabine administration for resected PDAC. The study involved 45 patients. The lymph node (LN) ratio and dCK protein expression were significant predictors of DFS and OS in univariate analysis. On multivariate analysis, a step-down procedure based on the likelihood ratio test, dCK protein expression was the only independent prognostic factor.

Having previously reported that Notch3 activation appeared to be associated with more aggressive PC disease, Mann *et al*[50] examined components of this pathway (Notch1, Notch3, Notch4, HES-1, HEY-1) in resectable and non-resectable tumors compared to uninvolved pancreas. All three Notch family members were significantly increased in tumor tissue, with expression maintained within matched lymph node metastases. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1 and HEY-1 was noted in locally advanced and metastatic tumors compared to resectable cancers. Nuclear Notch3 and HEY-1 expression were significantly associated with reduced OS and DFS following tumor resection.

Diagnostic biomarkers

Regarding diagnostic biomarkers (Table 2), the most recent studies are based on the determination of protein panels in serum or plasma, exploiting ELISA or proximity ligation assay (PLA) for their determination. PLA is a highly sensitive technique for multiplex detection of biomarkers in plasma with little interfering background signal. Some of the studies proposed the determination of both diagnostic and prognostic markers. All studies presented here involve CA19-9 as a potential biomarker in association with other markers.

In the first study, Chang *et al*[51] applied PLA to the identification of plasma levels of 21 biomarkers in 52 locally advanced PDAC cases and 43 age-matched controls. The optimal diagnostic biomarker panel was computed using a combination of the PAM algorithm and logistic regression modeling.

In the second study, Brand *et al*[52] investigated 83 circulating proteins in sera of patients diagnosed with PDAC, benign pancreatic conditions and healthy controls. Samples from each group were split randomly into training and blinded validation sets prior to analysis. A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set. Identified panels were evaluated in the validation set and in patients diagnosed with colon, lung and breast cancers.

In the third study, Schultz *et al*[53] tested the hypothesis that high plasma YKL-40 and IL-6 are associated with PC and short OS. 559 patients with PC from prospective biomarker studies were studied. Plasma YKL-40 and IL-6 were determined by ELISA and serum CA 19.9 by che-

miluminescent immunoassay.

Chang *et al*[51] found that three markers (CA19-9, OPN and CHI3L1) have superior sensitivity for PC *vs* CA19-9 alone (93% *vs* 80%) and two markers (CEA and CA125) proved to have a prognostic significance for survival of PC ($P < 0.003$) when measured simultaneously. Brand *et al*[52] found that the panel of CA 19-9, CEA and TIMP-1 discriminated PDAC patients from benign subjects with an SN/SP of 76%/90% in the training set and of 71%/89% in the validation set. The CA19-9, intercellular adhesion molecule 1 (ICAM-1), OPG panel is selective for PDAC and does not recognize breast (SP = 100%), lung (SP = 97%) or colon (SP = 97%) cancer. Schultz *et al*[53] instead showed that high preoperative IL-6 and CA 19.9 were independently associated with short OS. High pre-treatment levels of each biomarker were independently associated with short OS in non-operable patients.

PROTEOMIC BASED STUDIES

The most recent studies on identification of biomarkers in proteomics (Table 3) are mainly regarding the determination of diagnostic markers. The studies are presented separating those carried out from serum or tissue samples and those carried out on cell lines or animal models. The studies presented here are mainly devoted to identifying exploratory biomarkers.

The studies based on proteomic approaches show a great potential for the identification of PC biomarkers; the panels of markers identified by these techniques are characterized by good performance regarding both sensitivity and specificity, showing results at least equal to or in some cases better than classical approaches. It is the authors' opinion that, in future, the information provided by such high-throughput techniques should be coupled with clinical information to provide exhaustive sets of biomarkers with better predictive ability.

Diagnostic biomarkers in tissue and serum samples

Tissue and serum biomarkers in proteomics are usually determined by: SDS-PAGE followed by LC-MS for identifying the most up- or down-regulated proteins; matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry; and surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry. The exploitation of instrumental techniques providing a high amount of information makes the application of multivariate methods necessary in order to detect panels of biomarkers with the best predictive ability.

In the first study by McKinney *et al*[54], matched pairs of tumor and non-tumor pancreas from patients undergoing tumor resection were treated to obtain cytosol, membrane, nucleus and cytoskeleton cellular protein fractions. The fractions were analyzed by SDS-PAGE followed by LC-MS/MS to identify 2393 unique proteins. The spectral count data were compared using a power law global error model (PLGEM) to identify statistically significant protein changes between non-tumor and tumor samples[55,56]. Among the 104 proteins significantly changed in cancers, four (biglycan (BGN), pigment epithelium-derived factor (PEDF), thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3)) were further validated and proved to be up-regulated in cancer and have potential for development as minimally-invasive diagnostic markers.

Kojima *et al*[57] differentiated pancreatic neoplasia from non-neoplastic pancreatic disease. Samples from 50 patients [15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP)] were collected. A high-throughput method was applied, using high-affinity solid lipophilic extraction resins, enriched low molecular weight proteins for extraction with a high-speed MALDI-MS. Multivariate analysis was carried out by MDS as in Mobley *et al*[58]. Using eight serum features, Ca were differentiated from H (SN = 88%, SP = 93%), Ca from CP (SN = 88%, SP = 30%) and Ca from both H and CP combined (SN = 88%, SP = 66%). In addition, nine features obtained from urine differentiated Ca from both H and CP, combined with high efficiency (SN = 90%, SP = 90%). Interestingly, the plasma samples did not show significant differences.

Ehmann *et al*[59] and Hauskrecht *et al*[60] instead applied SELDI-TOF mass spectrometry. In the first study[59], 96 serum samples from patients undergoing cancer surgery were compared with 96 controls. Samples were fractionated by anion exchange chromatography. Data analysis, involving Mann-Whitney *U* test and classification and regression tree (CART) analysis, identified 24 differentially expressed protein peaks, 21 of which were under-expressed in cancer samples. The best single marker can predict 92% of controls and 89% of cancer samples. The best model with a set of 3 markers showed a sensitivity of 100% and a specificity of 98% for the training data and a sensitivity of 83% and a specificity of 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I were identified as markers (decreased in cancer sera). Hauskrecht *et al*[60] instead proposed a feature selection method that extracts useful feature panels from high-throughput spectra. 57 PC samples were compared to 59 controls. The results clearly show the improved classification performances when the method is compared to standard strategies.

The last study makes use of protein microarrays to explore whether a humoral response to PC-specific tumor antigens has utility as a biomarker of PC. To determine if such arrays can be used to identify novel autoantibodies in the sera from PC patients, Patwa *et al*[61] resolved proteins from a PDAC cell line (MIAPACA) by 2-D liquid-based separations and then arrayed them on nitrocellulose slides. The slides were probed with sera from a set of patients diagnosed with PC and compared with age- and sex-matched normal subjects. To account for patient-to-patient variability, a non-parametric Wilcoxon rank-sum test was used in which protein biomarkers were identified. Classification by the PAM algorithm showed 86.7% accuracy, with a SN and SP of 93.3% and 80%, respectively. The identified candidate autoantibody

Table 3 Proteomic based studies

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
54	D	Among 2393 unique proteins, 104 proteins significantly changed in cancer	T	5 patients; matched pairs of tumor and non-tumor pancreas	Tissues treated to obtain cytosol, membrane, nucleus and cytoskel-etonfractions. Fractions separated and digested underwent LC-MS/MS	PLGEM	104 proteins significantly changed in cancer. Among these, 4 proteins validated that were up-regulated in cancer: biglycan (BGN), Pigment Epithelium-derived Factor (PEDF) Thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3)
57	D	Serum MALDI-TOF features	S	15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP) samples	MALDI-TOF	Nonparametric	8 serum features: Ca samples differentiated from H (SN = 88%, SP = 93%), Ca from CP (SN = 88%, SP = 30%), and Ca from both H and CP combined (SN = 88%, SP = 66%). 9 features obtained from urine: differentiated Ca from both H and CP combined (SN = 90%, SP = 90%)
59	D	Serum SELDI-TOF features	S	96 serum samples from patients undergoing cancer surgery compared with sera from 96 controls	SELDI-TOF	pairwise statistics, MDS, hierarchical analysis Mann-Whitney *U* test, CART	Data analysis identified 24 differentially expressed protein peaks, 21 of which under-expressed in cancer samples. The best single marker predicts 92% of controls and 89% of cancer samples. Multivariate analysis: best model (3 markers) with SN = 100% and SP = 98% for the training data and SN = 83% and SP = 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I identified as markers and decreased at least 2 fold in cancer sera
60	D	Serum SELDI-TOF features	S	57 PC samples were compared to 59 controls	SELDI-TOF	Multivariate decorrelation filtering	Improved classification performances when the presented strategy is compared to standard univariate feature selection strategies
61	D	Proteins	S	Sera from patients diagnosed with PC compared with age- and sex-matched normal subjects	Protein microarrays	Rank-based non-parametric statistical testing	A serum diagnosis of PC was predicted with 86.7% accuracy, with a sensitivity and specificity of 93.3% and 80%. Candidate autoantibody biomarkers studied for their classification power using an independent sample set of 238 sera. Phosphoglycerate kinase-1 and histone H4 noted to elicit a significant differential humoral response in cancer sera compared with age- and sex-matched sera from normal patients and patients with chronic pancreatitis and diabetes
62	D	Proteins	PDAC cell lines	435 spots identified from 18 samples from 2 cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells	2D-PAGE	PCA, SIMCA, Ranking-PCA	Samples were all perfectly classified. Significant proteins were further identified by MS analysis
63	D	Proteins regulating the conversion of quiescent to activated PaSC cells	rat PaSC cell line	-	SDS-PAGE and GeLC-MS/MS	QSPEC	Qualitative and quantitative proteomic analysis revealed several hundred proteins as differentially abundant between the two cell states. Proteins of greater abundance in activated PaSC: isoforms of actin and ribosomal proteins. Proteins more abundant in non-proliferating PaSC: signaling proteins MAP kinase 3 and Ras-related proteins

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample: S: Serum; P: Plasma; T: Tissue.

biomarkers were validated using an independent sample set of 238 samples. Phosphoglycerate kinase-1 and histone H4 were noted to elicit a significant differential humoral response in cancer sera.

Diagnostic biomarkers from cell lines or animal model samples

Diagnostic biomarkers in proteomics have also been recently determined from cell lines (PACA44, T3M4) or on animal models. The most exploited analytical techniques in this case are SDS-PAGE, followed by LC-MS, 2D-PAGE or 2D-LC approaches.

Marengo *et al*[62] identified the regulatory proteins in human PC treated with trichostatin A by 2D-PAGE maps and multivariate analysis. PCA was applied to a spot quantity

dataset comprising 435 spots detected in 18 samples belonging to two different cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells. PCA allowed the identification of the groups of samples present in the dataset; the loadings analysis allowed the identification of the differentially expressed spots, which characterize each group of samples. The treatment of both the cell lines with trichostatin A showed an evident effect on the proteomic pattern of the treated samples. Identification of some of the most relevant spots was also performed by MS analysis. The same authors applied different multivariate statistical tools to the same set of data to provide sets of candidate biomarkers; the first application regards the exploitation of SIMCA classification to evaluate the biomarkers characterized by a significant discriminant power[25], while the second application regards the development of ranking PCA[28]. This second application is particularly interesting for overcoming the limitations of the methods usually adopted as variable selection tools to identify only significant biomarkers: they are usually aimed at the selection of the smallest set of variables (spots) providing the best performances in prediction. This approach does not seems to be the best choice in the identification of potential biomarkers since all the possible candidate biomarkers have to be identified to provide a general picture of the "pathological state"; exhaustivity has to be preferred to provide a complete understanding of the mechanisms underlying the pathology. Ranking PCA allowed the exhaustive identification of a complete set of candidate biomarkers.

Paulo *et al*[63] compared differentially expressed proteins in rat in activated and serum-starved non-proliferating pancreatic stellate cells (PaSC), emerging key mediators in chronic pancreatitis and PC pathogenesis. About 1500 proteins were identified after SDS-PAGE and LC-MS/MS. Qualitative and quantitative proteomic analysis revealed several hundred proteins to be differentially abundant between the two cell states. Significance analysis was performed using QSPEC, a recently published algorithm for determining the statistical significance of differences in spectral counting data from two sample sets[64]. This algorithm exploits the Bayes factor instead of the *P*-value as a measure of statistical significance[65,66]. Proteins of greater abundance in activated PaSC included isoforms of actin (*e.g.,* smooth muscle actin) and ribosomal proteins. Proteins more abundant in non-proliferating PaSC than in activated PaSC included signaling protein MAPK-3 and Ras-related proteins. The molecular functions and biological pathways for these proteins were also determined by gene ontology analysis and KEGG pathway.

Other studies based on a proteomic approach

Paulo *et al*[67] also evaluated the endoscopic pancreatic function test (ePFT) as a method able to safely obtain pancreatic fluid for MS analysis from patients during upper endoscopy and reproducibly identify pancreas-specific proteins. The ePFT-collected pancreatic fluid from 3 individuals without evidence of chronic pancreatitis was analyzed by SDS-PAGE and GeLC-MS/MS. The SDS-PAGE analysis revealed no significant variation in protein concentration during the 1 h collection. The GeLC-MS/MS analysis identified pancreas-specific proteins previously described from endoscopic retrograde cholangiopancreatography and surgical collection methods. Gene ontology further revealed that most of the proteins identified have a molecular function of proteases.

GENOMIC-BASED STUDIES

The most recent studies on identification of biomarkers in genomics (Table 4) regard both the determination of prognostic/predictive markers and diagnostic markers and are presented hereafter separated into these two classes. The studies presented here are mainly devoted to identifying exploratory biomarkers.

The studies based on genomic approaches have potential to identify PC biomarkers. In future, the information provided by genomic approaches should be coupled to proteomic, metabolomic and clinical information to improve the predictive ability of the panels of identified biomarkers.

Prognostic and/or predictive biomarkers

Prognostic and predictive biomarkers in genomics (Table 4) are usually determined in tissue samples. Some of the proposed studies include both protein expression and microRNA expression profiles. In these studies, multivariate Cox regression analysis is usually exploited.

Ogura *et al*[68] studied the K-ras mutation status in 242 patients with unresectable PC. The authors focussed on K-ras mutation subtypes since recent reports indicate that K-ras mutation status acts as a prognostic factor. CA19-9, metastatic stage and mutant-K-ras were negative prognostic factors, indicating a reduced survival. Among the patients who had K-ras mutation subtypes, CA19-9, metastatic stage and the presence of the G12D or G12R mutations were negative prognostic factors for OS.

Hwang *et al*[69] evaluated whether expression of novel candidate biomarkers, including microRNAs, can predict clinical outcome in PDAC patients treated with adjuvant therapy. 82 resected PDAC cases were analyzed for protein expression by immunohistochemistry and for microRNA expression by quantitative real time PCR (qRT-PCR). Lower than median miR-21 expression was associated with a significantly lower HR for death and recurrence in the subgroup of patients treated with adjuvant therapy. MiR-21 expression status emerged as the single most predictive biomarker for treatment outcome. No significant association was detected in patients not treated with adjuvant therapy. The results were confirmed in an independent validation of 45 PDAC tissues.

One-fifth of patients with seemingly "curable" PDAC experienced an early recurrence and death, while

Table 4 Genomic based studies

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
68	P	*K*-ras mutation status and subtypes	endoscopic ultrasound-guided fine-needle aspiration specimens	242 patients	RT-PCR	Kaplan Meier method and Cox proportional hazards regression	Multivariate analysis: CA19-9 C 1000 U/mL (HR = 1.78, 95 %CI: 1.28-2.46, $P < 0.01$), metastatic stage (HR 2.26, 95 % CI 1.58-3.24, $P < 0.01$) and mutant-K-ras (HR 1.76, 95 %CI: 1.03-3.01, $P = 0.04$) negative prognostic factors. Among patients with K-ras mutation subtypes: CA19-9 C 1000 U/mL (HR 1.65, 95%CI: 1.12-2.37, $P < 0.01$), metastatic stage (HR 2.12, 95%CI: 1.44-3.14, $P < 0.01$), and G12D or G12R mutations (HR = 1.60, 95%CI: 1.11-2.28) negative prognostic factors for OS
69	P	MicroRNA-21	T	82 resected Korean PDAC cases. Subgroup of patients treated with adjuvant therapy ($n = 52$)	Protein expression by immunohistochemistry microRNA expression by qRT-PCR	Cox proportional hazards model	Subgroup with adjuvant therapy: lower than median miR-21 expression associated with lower HR for death (HR = 0.316, 95%CI = 0.166-0.600, $P = 0.0004$) and recurrence (HR = 0.521, 95%CI = 0.280-0.967, $P = 0.04$). MiR-21: single most predictive biomarker for treatment outcome. No significant association in patients not treated with adjuvant therapy. Independent validation cohort of 45 frozen PDAC tissues from Italian cases treated with adjuvant therapy: lower than median miR-21 expression confirmed to be correlated with longer OS and DFS
71	P	13 putative PDA biomarkers from the public biomarker repository		A survival tissue microarray was constructed comprised of short-term (cancer-specific death ,12 mo, $n = 58$) and long-term survivors (30 mo, $n = 79$) who underwent resection for PDA (total, $n = 137$)	Immunohistochemical analyses; survival tissue microarray (s-TMA)	Wilcoxon rank sum test	Multivariate model: MUC1 (OR = 28.95, 3+ *vs* negative expression, $P = 0.004$) and MSLN (OR = 12.47, 3+ *vs* negative expression, $P = 0.01$) highly predictive of early cancer-specific death. Pathological factors (size, lymph node metastases, resection margin status, and grade): ORs < 3 and none reached statistical significance. ROC curves used to compare the 4 pathological prognostic features (ROC area = 0.70) to 3 univariate molecular predictors (MUC1, MSLN, MUC2) of survival group (ROC area = 0.80, $P = 0.07$)
1	P	MTA2 mRNA and protein expression	T	123 PDAC samples and 40 control tissues	qRT-PCR and immunohistochemistry	Kaplan-Meyer curves and Cox analysis	MTA2 mRNA and protein expression levels up-regulated in PC. MTA2 correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS. MTA2: independent prognostic factor.
72	D	Leukocyte DNA Methylation	Blood	Phase I: 132 never-smoker PaC patients and 60 never-smoker healthy controls. Phase Ⅱ: validation of 88 of 96 phase Ⅰ selected CpG sites in 240 PaC cases and 240 matched controls	DNA array	Wilcoxon Rank Sum tests and likelihood penalized logistic regression models	Significant differences found in 110 CpG sites (FDR < 0.05). Phase Ⅱ: 88 of 96 phase Ⅰ selected CpG sites validated in 240 PaC cases and 240 matched controls ($P < 0.05$). Prediction model: 5 CpG sites (IL10_P348, LCN2_P86, ZAP70_P220, AIM2_P624, TAL1_P817) discriminated PaC from controls (C = 0.85 in phase I; 0.76 in phase Ⅱ). One CpG site (LCN2_P86) could discriminate resectable patients from controls (C = 0.78 in phase I; 0.74 in phase Ⅱ). 3 CpG sites identified (AGXT_P180_F, ALOX12_E85_R, JAK3_P1075_R) where the methylation levels were significantly associated with SNPs (FDR < 0.05)
73	D	cell-surface targets	T	28 PC specimens and 4 normal pancreas tissue samples. Expression in normal tissues evaluated by array data from 103 samples representing 28 organ sites as well as mining published data	Complementary assays of mRNA expression. Immunohistochemistry. qRT-PCR	-	170 unique targets highly expressed in 2 or more PC specimens and not expressed in normal pancreas samples. Two targets (TLR2 and ABCC3) further validated for protein expression by tissue microarray based immunohistochemistry have potential for the development of diagnostic imaging and therapeutic agents for PC
74	D	Differentially expressed genes	Blood	25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 yr, and 25 healthy controls. Results further validated for 101 blood samples	Microarray and qRT-PCR	-	58 genes found to be unique in patients with cancer-associated diabetes, including 23 up-regulated and 35 down-regulated genes. 11 up-regulated genes further validated by RT-PCR; 2 of these (VNN1 and MMP9) selected for logistic regression analysis. The combination of VNN1 and MMP9 showed the best discrimination of cancer-associated diabetes from type 2 diabetes. The protein expression of MMP9 and VNN1 was in accordance with the gene expression

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample: S: Serum; P: Plasma; T: Tissue; PDAC: Pancreatic ductal adenocarcinoma; VNN1: Vanin-1; MMP9: Matrix metalloproteinase 9.

some patients with advanced stage tumors are deemed "unresectable" by conventional staging criteria, yet progress slowly. Effective biomarkers that stratify PDAC based on the biological behavior are therefore needed. Building on a compendium of 2500 published candidate biomarkers in PDAC[70], Winter *et al*[71] constructed a survival tissue microarray (s-TMA) comprised of short-term (12 mo) and long-term survivors (30 mo) who underwent resection for PDAC. The s-TMA acts as a biological filter to identify prognostic markers. 13 putative PDAC biomarkers were identified from the public biomarker repository and tested against the s-TMA. MUC1 and MSLN were highly predictive of early cancer-specific death. By comparison, no pathological factors (size, lymph node metastases, resection margin status and grade) reached statistical significance.

Chen *et al*[1] detected metastasis-associated gene 2 (MTA2) expression in PDAC and related it to prognosis. MTA2 mRNA and protein expression were determined by qRT-PCR and immunohistochemistry in primary cancers and their adjacent non-cancerous tissues. MTA2 mRNA and protein expression levels were up-regulated in PC. MTA2 was correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS.

Diagnostic biomarkers

Diagnostic biomarkers from genomics-based studies (Table 4) are identified both from tissue and serum samples.

To identify biomarkers for early detection, Pedersen *et al*[72] examined DNA methylation differences in leukocyte DNA between PC cases and controls. In phase I, methylation levels were measured at 1505 CpG sites in leukocyte DNA from 132 never-smoker PC patients and 60 never-smoker controls. Significant differences were found in 110 CpG sites. In phase II, 88 of 96 phase I selected CpG sites were tested and validated in 240 PC cases and 240 matched controls. Using penalized logistic regression, a prediction model was built consisting of five CpG sites (IL10_P348, LCN2_P86, ZAP70_P220, AIM2_P624, TAL1_P817) that discriminated cancer patients from controls. One CpG site (LCN2_P86) alone could discriminate resectable patients from controls.

Morse *et al*[73] used complementary assays of mRNA expression profiling of cell-surface genes to determine increased expression in PC *vs* normal pancreas tissues and validated protein expression by immunohistochemistry on tissue microarrays. This approach was aimed at the identification of targets for potential use in the molecular imaging of cancer, allowing for non-invasive determination of tumor therapeutic response and molecular characterization of the disease, or in the targeted delivery of therapy to tumor cells, decreasing systemic effects. Expression profiles of 2177 cell-surface genes for 28 pancreatic tumor specimens and 4 controls were evaluated. 170 unique targets were highly expressed in 2 or more of the pancreatic tumor specimens and were not expressed in controls. Two targets (TLR2 and ABCC3) were further validated for protein expression and proved to be potential for the development of diagnostic imaging and therapeutic agents for PC.

Huang *et al*[74] explored specific biomarkers that can differentiate PC-associated diabetes from type 2 diabetes for the early detection of PC. Peripheral blood samples were collected from 25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 years, and 25 controls. 32 samples were used in microarray experiments to find differentially expressed genes specific for cancer-associated diabetes. The results were further validated by quantitative qRT-PCR for 101 blood samples. Protein expression of selected genes in serum and tissues was also detected. 58 genes were found to be unique in patients with cancer-associated diabetes (23 up-regulated; 35 down-regulated). 11 up-regulated genes were further validated by RT-PCR and 2 of these, vanin-1 (*VNN1*) and matrix metalloproteinase 9 (*MMP9*), showed the best discrimination of cancer-associated from type 2 diabetes.

METABOLOMICS BASED STUDIES

Just one paper, by Kaur *et al*[75], has recently appeared in the literature, reporting for the first time the mass spectrometry-based metabolomic profiling of human pancreas in matched tumor and normal tissues. UPLC coupled with TOF-MS was applied to perform small molecule metabolite profiling of matched normal and PC tissues. The resulting multivariate data matrix was pre-processed for spectral alignment and peak detection, followed by normalization of the data to the feature intensities of the internal standard as well as to the total protein concentration. The normalized data were analyzed first by PCA, followed by OPLS[31]. The authors also exploited random forest clustering[32] to interrogate the top 50 features with significant alterations in the tumor tissue compared to the control. The candidate markers were searched against different databases[76,77] to find compounds that corresponded to the accurate monoisotopic mass measurements detected by UPLC-TOFMS analysis. The authors report a subset of metabolites which were unequivocally identified and found to be significantly de-regulated in PC tissues.

The study reported here proves that metabolomic profiling shows great potential for the identification of biomarkers for PC. Certainly, further characterization and validation with a large sample size is needed and may help establish the utility of such markers as biomarkers of clinical benefit.

CONCLUSION

This review aimed to present the most recent applications of the omics approaches (proteomics, genomics and metabolomics) to the identification of biomarkers for PC. Particular attention has been paid to the statistical methods adopted for identification of biomarkers, first

presenting the main statistical procedures adopted from a theoretical point of view. Then, the most recent applications present in the literature were presented separately for non-omic, proteomic, genomic and metabolomic based studies. Within this distinction, studies were presented separately for diagnostic and prognostic/predictive biomarkers and according to the type of marker.

Different statistical approaches are exploited in the literature for the identification of markers in PC; the methodologies presented here appear to be effective and sound. However, it is the authors' opinion that multivariate methods have to be preferred. With the term multivariate, the authors refer to methods evaluating the relationships between the variables (both predictors and outcomes if several of both are present) in order to provide a pool of markers highlighting synergistic and antagonistic effects. In fact, the biological effect played by pathology (and PC makes no exception) is the result of a series of different mechanisms independent from each other or showing relevant interactions. Among all strategies, therefore, multivariate ones able to point out these relationships are preferred.

Another hint that must be addressed is the risk of identifying false positives, *i.e.*, markers erroneously identified as such; this risk greatly increases when little information is available (*i.e.*, a small number of cases/patients is investigated). Certainly, this problem is deeply related to the problem of experimental design and sample collection and each study should be carefully designed from a statistical point of view before being performed in order to include all possible sources of biological variation. Of course, this necessity often clashes with the availability of samples, especially when tissue collection is involved. From a statistical point of view, in these cases characterized by little information, it is very important to apply mathematical tools to validate the models built, thus evaluating the predictive ability of the models. In this respect, the use of cross-validation techniques or simulation algorithms is fundamental to identify only statistically significant markers.

Certainly, there is a great gap between the results presented in studies on the identification of candidate markers reported in literature and the actual possibility of exploiting the identified biomarkers at a clinical level. This is due to several aspects, among which the most important are the poor sensitivity/specificity sometimes characterizing the identified pools of markers and the complicated biostatistic design of prospective studies for their validation prior to clinical use.

It is the authors' opinion that the future perspective in exploratory identification of biomarkers has to be found in the exhaustive search for potential markers. It is impossible to imagine that complex pathology acting on a wide range of individuals characterized by a large biological variability could be reflected in a very restricted panel of markers. We think that the future will rely on high-throughput techniques and the possibility of combining the results emerging from proteomic, genomic, metabolomic studies coupled with clinical information to identify exhaustive panels of markers, thus improving the predictive performance of the panels themselves and providing better sensitivity and specificity. Certainly, great attention has to be paid in such studies to the proper evaluation of experimental and biological variability (*i.e.*, a careful selection of experimental design) to provide sound and robust results and to the evaluation of the results through effective multivariate techniques.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Gene therapy in pancreatic cancer

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Abstract

Pancreatic cancer (PC) is a highly lethal disease and notoriously difficult to treat. Only a small proportion of PC patients are eligible for surgical resection, whilst conventional chemoradiotherapy only has a modest effect with substantial toxicity. Gene therapy has become a new widely investigated therapeutic approach for PC. This article reviews the basic rationale, gene delivery methods, therapeutic targets and developments of laboratory research and clinical trials in gene therapy of PC by searching the literature published in English using the PubMed database and analyzing clinical trials registered on the Gene Therapy Clinical Trials Worldwide website (http://www. wiley.co.uk/genmed/ clinical). Viral vectors are main gene delivery tools in gene therapy of cancer, and especially, oncolytic virus shows brighter prospect due to its tumor-targeting property. Efficient therapeutic targets for gene therapy include tumor suppressor gene *p53*, mutant oncogene *K-ras*, anti-angiogenesis gene *VEGFR*, suicide gene *HSK-TK*, cytosine deaminase and cytochrome *p450*, multiple cytokine genes and so on. Combining different targets or combination strategies with traditional chemoradiotherapy may be a more effective approach to improve the efficacy of cancer gene therapy. Cancer gene therapy is not yet applied in clinical practice, but basic and clinical studies have demonstrated its safety and clinical benefits. Gene therapy will be a new and promising field for the treatment of PC.

Key words: Pancreatic cancer; Gene therapy; Tumor suppressor; Suicide; Anti-angiogenesis; Immunotherapy; Oncogene; Multidrug resistance; Clinical trial

Core tip: This paper tries to present a full picture of gene therapy in pancreatic cancer, providing an unambiguous classification and comprehensive analysis, especially in therapeutic targets and clinical trials worldwide. From our work, you may find the hotspots in related research and the reason why they get there.

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INTRODUCTION

Pancreatic cancer (PC) is an aggressive and highly lethal malignant disease. The incidence of PC is lower than that of many other types of cancer, but it is the fourth most common cause of death from cancer[1]. PC is highly malignant and invasive, owing to nonspecific incipient symptoms and early metastasis. Most patients have local or metastatic spread at the time of presentation, and less than 15% of patients are candidates for surgery. Therefore, the prognosis of the disease remains poor. Recent statistics from the US National Cancer Institute showed that the overall 5-year relative survival rate for 2002-2008 was 5.8%, and nearly 90% of all patients were dead in 1

year from diagnosis, with a median survival less than 6 mo[2,3].

Surgery is still the first line treatment for PC, because it provides the only curable option. Other adjuvant treatments are chemotherapy, radiotherapy, physiotherapy and biotherapy. However, PC is highly resistant to the currently available chemotherapy and radiotherapy, and it is one of the cancers for which survival rate has not been substantially improved during the past 30 years. Therefore, new effective modalities for the treatment of this disease are urgently required. In recent years, with the outstanding progress of modern molecular biology, tumor immunology and gene engineering technology, tumor biotherapy is becoming a perspective and rapidly developing field of modern medicine, which is expected to improve state of or even cure patients who are not curable by classical methods of therapy.

Generally, tumor biotherapy includes immunotherapy and gene therapy, but there is no explicit boundary between them. Gene therapy can be used to transfer genes into tumor cells to render them more highly immunogenic, while cancer immunotherapy utilizes gene engineering technology to produce immunomodulating agents, such as tumor vaccines.

Since the first gene therapy clinical trial was approved by the National Institutes of Health in May 1990, significant progress in gene therapy technology has been achieved. In September 2006, a successful immunogene therapy of two patients with metastatic melanoma was reported. Up to July 2013, we have entries for 1970 trials undertaken in 31 countries, and most of them had been aimed at the treatment of cancer (64.2% of all gene therapy trials)[4]. In 2008, detailed, global, genomic analyses found that PC contained an average of 63 genetic alterations, the majority of which were point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67%-100% of the tumors[5]. PC gene therapy is, therefore, targeting genes involved in these cellular pathways, inducing direct cell apoptosis and/or stimulating host immune defense system against tumor growth and expansion. Highly efficient gene therapy regimen is based on the following key points: efficiency of gene delivery, tumor targeted therapy and selection of efficient targets.

STRATEGIES FOR GENE THERAPY

Gene replacement

This strategy seeks chances of replacing a mutated gene with a normal gene *via in situ* homologous recombination. It is the best way to treat or even cure monogenic diseases, but seldom used for cancer gene therapy because of technical limitations and complex genetic alterations in cancer.

Gene modification

This strategy tries to directly modify the mutated gene and rehabilitate functions of target cells. It is an ideal manner of gene therapy but with great difficulties. Rare research related to this strategy has been reported.

Gene augmentation

Gene augmentation intends to transfer exogenous therapeutic genes into deficient cells and let their expression products make up for the deficiency. This is the most commonly used strategy in gene therapy. Key point of this technology is the selection of therapeutic genes and gene delivery systems. Plenty of efficient delivery systems have been developed to introduce genetic material into eukaryotic cells and get them expressed. The details will be discussed below.

Gene blockade

This strategy seeks to prevent the transcription and translation of certain cancer-associated genes by using short nucleotide sequences that bind in a complementary fashion to specific DNA or RNA, which can block aberrant signal transduction pathway and induce tumor differentiation and apoptosis eventually. It is also known as antisense gene therapy. Common materials used in this strategy include antisense oligonucleotides, ribozymes and small interfering RNAs (siRNAs).

Antisense oligonucleotides: Antisense oligonucleotides are short single-stranded segments of DNA or RNA artificially synthesized *in vitro*, which can selectively inhibit the transcription and translation of the target gene through the Watson-Crick base pairing between the antisense nucleotide and the target RNA or DNA. Since DNA is more easily synthesized and stable in body fluid, antisense oligodeoxyribonucleotides have become the most common material used in practice. Various chemical modifications to its backbone have been used to improve oligonucleotide stability, targeting and transduction efficiency[6].

Ribozymes: Ribozymes are RNA molecules with catalytic activity that are capable of sequence specific cleaving of mRNA molecules. They can selectively bind to target mRNAs through the Watson-Crick base pairing and form a duplex, which includes a highly distorted conformation that is easily hydrolyzed. The hydrolysis of the mRNA can be used for targeted suppression of specific genes[6].

However, a shortcoming of ribozymes is that their RNA backbone makes them easy targets for degradation by the ubiquitous RNAases, so these molecules are biologically unstable *in vivo*. Then researchers found another category of ribozymes, which are called DNAzymes or deoxyribozymes. They are analogs of ribozymes with greater biological stability, employing DNA motifs to replace the RNA backbone. Moreover, these DNAzymes are also easy to modify synthetically, thereby generating even stronger, resilient second-generation analogs, which makes them powerful tools for gene suppression applications[6].

siRNAs: siRNAs are short double-stranded RNA seg-

ments with typically 21- to 23-nucleotide bases that are complementary to the target mRNA sequence. siRNAs can be artificially synthesized *in vitro* and directly transferred into target cells, or be produced in the genetically modified target cells, in which a gene encoding siRNA is introduced *via* appropriate vectors, with the help of endogenous RNAase. When entering into the target cell, siRNAs bind to ribozyme compounds and form RNA-induced silencing complexes (RISCs), which bind to the target mRNA and stimulate mRNA degradation mechanisms, such as nuclease activity, that lead to silencing of the particular gene. Compared with other gene blockade technologies, siRNAs are remarkably superior because of their high degree of specificity to mRNAs, nonimmunogenic nature and high resistance to ribonucleases. Since siRNAs do not integrate into the genome, they offer greater safety than plasmid molecules. Furthermore, siRNAs do not have to transfer through the nuclear membrane and therefore require less sophisticated delivery systems, promising faster development and higher efficiencies[6]. Thanks to these advantages, RNA interfering technique has become one of the hotspots in research of gene therapy.

METHODS FOR GENE DELIVERY

Ex vivo delivery

In this system, the recipient cells which are previously explanted from the target tissue or bone marrow are cultured or proliferated *in vitro* and subsequently reinfused into the patient after therapeutic gene transfer. Obviously, only transplantable cells, such as lymphocytes and medullary cells, are acceptable in this method. In cancer therapy, tumor cells can also be cultured and engineered *in vitro*, but usually they are used to secrete cytokines or act as a vaccine. To improve the therapeutic efficacy, positively transfected cells are screened from the total cells for implantation, which gives *ex vivo* delivery higher transduction efficiency than *in vivo* delivery. However, the shortcomings of *ex vivo* delivery are complex operational process and a low survival rate of reimplanted cells[7,8].

In vivo delivery

In this system, gene vectors carrying therapeutic genes are directly delivered into the target tissues or organs, *via* systemic injection, *in situ* injection, oral agents or spray, of which *in situ* injection into local tumor tissue mediated by imaging methods is the most commonly used and ripest technology. Almost all the clinical trials on *in vivo* cancer gene therapy are based on this method, which includes intratumoral injection mediated by CT or ultrasound, tumor main vascular perfusion and gene-eluting stent implantation.

In vivo delivery is superior for its simple operation, easy preparation, independence on cell culture systems and wide range of application, whereas low efficiency of transduction, short curative effect, poor target cell specificity and immunologic problems are the main problems of this system. *In vivo* delivery might be the most useful strategy in clinical application. If only we overcome the shortcomings of this technique, gene therapy can truly be widespread applied in clinical treatment[9].

VECTOR SYSTEMS FOR GENE DELIVERY

The core problem on whether we choose *in vivo* delivery or *ex vivo* delivery is how to achieve specific gene transfection and highly efficient gene expression in recipient cells. As a consequence, establishing an efficient, safe and specialized delivery system has become the foundation of gene therapy. An ideal gene delivery system should have these characters: (1) non-invasive mode of administration; (2) tumor-specific targeting, including primary lesion and distant metastatic lesion, especially site specific lesion, such as the central nervous system and testis; (3) sustained gene expression; and (4) high insertion capacity, bio-safety, stability and easy preparation.

These vector systems can be divided into two categories: non-viral and viral vector systems. Both of them have been investigated and each of them presents distinct advantages and weaknesses. Viral methods normally offer higher transduction efficiency and long-term gene expression, but it may be associated with toxicity, immunogenicity, mutagenicity, inability to transfer large size genes and high costs. Non-viral methods provide advantages including relative safety, ability to transfer large size genes, less toxicity and easy preparation; they can also be modified with ligands for tissue or cell specific targeting. However, non-viral methods show limitations of low transfection efficiency and poor transgene expression[10,11].

Non-viral vector systems

Non-viral vectors consist of chemical vectors, biological vectors and physical methods of gene transfer to introduce naked DNA (in the form of plasmid DNA), RNA molecules, or oligonucleotides into recipient cells.

Physical delivery

Physical delivery mainly includes microinjection, microparticle bombardment and electroporation.

Microinjection involves the utilization of a micropipette to inject nucleic acid directly into a single living cell at a microscopic level. It is highly efficient since one cell at a time is targeted for DNA transfer. However, this precision is achieved at the expense of time. Microparticle bombardment, also known as ballistic DNA injection, gene gun technology or DNA-coated particle bombardment, is used to transfer plasmid DNA coated with heavy metals, usually gold, tungsten or silver, which are used as payload. These particles can be accelerated by pressurized gas and fire at the target cells or tissues without injuring them. Nevertheless, since direct exposure of target tissues is required, its application is restricted in internal organs. In addition, low efficiency of transfection into the nucleus and plasmid DNA integration into host genome are also problems to be solved. Electroporation

uses high-voltage electrical current to generate transient disruption on the membrane of target cells, which allows the entry of plasmid DNA by diffusion. This technique results in high cell mortality and therefore is not suitable for clinical use.

In conclusion, though significant transfection efficiencies have been achieved using physical techniques, they are extremely difficult to standardize in a clinical setting and are considered laborious, impractical, and invasive[11].

Chemical vectors

Commonly used chemical vectors can be classified into two major types based on the nature of the synthetic material, including cationic lipids and cationic polymers. In recent years, chemical vectors have been widely studied due to their advantages, including safety, large size gene transfer ability, less toxicity, low cost and easiness in preparation.

Cationic lipids (liposomes) are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer[6]. DNA is bound by cationic lipids as a result of electrostatic interaction, which allows for fusion of the liposome with the target cell membrane, endocytosis, and delivery of the DNA into the cytoplasm. Cationic lipids mediated gene transfer is the most promising method in non-viral delivery systems. Although this approach has already been applied in clinical trials, some problems still need to be solved for its better application, including the toxicity and lower transfection efficiency *in vivo*[11].

Cationic polymer is an umbrella term of a wide range of chemical compounds, including: (1) natural polymers such as chitosan; (2) dendrimers such as polyamidoamine (PAMAM); (3) polypeptides such as poly-L-lysine (PLL), polyarginine, polyornithine, histones and protamines; and (4) other polymers such as polyethylenimine (PEI) and polyphosphoester[11]. Their transfection activity and toxicity vary dramatically. When mixed with negatively-charged DNA, positive charges of the polymers allow the formation of polymer/DNA complexes (polyplexes) through electrostatic interaction. Polyplexes are nano-sized transfection units that normally have higher stability than lipoplexes. The contributions of cationic polymers are enhancing the DNA uptake *via* endocytosis, protecting DNA from nuclease degradation and facilitating DNA escape from endosomes. Finally, DNA is released into the cytoplasm and migrates into the nucleus in which transgene expression takes place.

Recently, a combination of cationic polymers with liposomes, called polymer/lipid hybrid system, has also been developed and showed some superiority. To prepare this 3-part (lipid/polymer/DNA) system, DNA is precondensed by cationic polymers, followed by the subsequent complexation with liposomes. Using cationic lipids, the polymer/DNA complexes can be further condensed and protected, which can facilitate endocytosis and increase circulating half-life *in vivo*[11].

Furthermore, to improve the tissue or cell specificity of chemical vectors, they are also manipulated with the supplement of ligands or fusogenic peptides, such as transferrin, lectin and epidermal growth factor, which can bind to the receptors on the surface of target cells specifically. This approach is also known as receptor-directed gene transfer.

Biological vectors

Bacteria can be used as gene therapy vectors. When engineered to express the therapeutic transgene, bacteria can introduce both the therapeutic gene and protein product to recipient cells. The types of bacteria used include attenuated strains of *Salmonella*, *Shigella*, *Listeria*, and *Yersinia*, as well as non-pathogenic *Escherichia coli*. For some of these vectors, the mechanism of DNA transfer from the bacteria to the mammalian cell is not yet fully understood, but their potential to deliver therapeutic molecules has been demonstrated *in vitro* and *in vivo* in experimental models[12]. A bacterial cancer vaccine for pancreatic cancer - a live attenuated Listeria strain expressing mesothelin - has entered early-phase clinical trial and demonstrated antitumor effects[13].

In addition, many mammalian cell types can be used as carriers of gene therapy vectors, such as hematological cells and mesenchymal stem cells (MSCs)[14]. MSCs possess natural tropism towards tumors, making them a vehicle for targeted delivery of therapeutic genes into tumors. Many experiments have identified their significant antitumor effects *in vitro* and *in vivo*. However, to effectively use this therapeutic strategy in clinic, we still have to solve a number of technical problems[15].

Viral vector systems

Viral vectors: Viral vectors are the most commonly studied and applied gene delivery systems. More than two-thirds of clinical trials of gene therapy reported are viral therapies. These viruses can use their innate mechanism of infection to enter the cell and transfer DNA molecules into cells without any physical or chemical processing. The therapeutic gene then enters the nucleus, integrates into the host gene pool, and is eventually expressed.

The most common viral vectors in cancer gene therapy are adenovirus (AdV), retrovirus (RV), adeno-associated virus (AAV), lentivirus, herpes simplex virus (HSV), influenza virus, Newcastle disease virus, pox virus, and Epstein-Barr virus (EBV). Both advantages and disadvantages should be considered when selecting a viral vector, including insertion capacity, host range of infection, state of integration into host genome, efficiency of transfection and expression, immunogenicity, bio-safety and difficulty of preparation. The comparison of common viral vectors is shown in Table 1[6,9,16-18].

Oncolytic virus: Referring to viral treatment, there is another related field called oncolytic virotherapy in tumor-targeted gene therapy, which is an emerging treatment modality that uses replication-selective virus (or conditionally replicating virus) to destroy cancers. These natural viruses are genetically modified to be non-pathogenic

Table 1 Comparison of characters of common viral vectors

Virus	Viral genome	Insertion capacity	Host range of infection	State of integration into host genome	Efficiency of gene transfection and expression	Immunogenicity	Titers of preparation *in vitro* (PFU/mL)	Bio-safety
AdV	Double-stranded DNA	38 kb	Broad spectrum (both dividing and non-dividing cells)	No integration	High	High	10^{11}-10^{12}	Safe
AAV	Single-stranded DNA	4.9 kb	Broad spectrum	Site-specific integration on chromosome 19q13.3	High	Low	10^{12}, dependent on helper virus	Safe
RV	Single-stranded RNA	8 kb	Dividing cells only	Integrate randomly	Low	Low	10^{6}-10^{7}	Risk of insertional mutagenesis
Lentivirus	Single-stranded RNA	8 kb	Broad spectrum	Integrate randomly	High	Low	10^{9}-10^{10}	Risk of viral infection and insertional mutagenesis
Pox virus	Souble-stranded DNA	25 kb	Broad spectrum	No integration	High	High, function as immunologic adjuvant	10^{6}-10^{7}	Safe
HSV	Double-stranded DNA	15-30 kb	Nerve cells and epithelial cells, especially neuro- tropic speciality	No integration	High	Moderate	10^{11}-10^{12}	Risk of viral infection

but selectively infectious and cytotoxic to cancer cells. Viruses hijack the host cell's protein factory, disabling its production in favor of viral products, which are intrinsically cytotoxic. The infected host cell eventually lyses, releasing new virions capable of infecting other cells in a "bystander" effect, amplifying and propagating the initial effect of infection. These viruses can also evoke an immune response in the host, but in a tolerable morbidity. In conclusion, two main characteristics of oncolytic viruses are: (1) they replicate selectively in cancer cells and have self-amplification properties; and (2) they have cancer-cell-specific toxicity.

Meanwhile, these oncolytic viruses can also be engineered to carry exogenous genetic materials to produce therapeutic effects such as secreting cytokines and enhancing antitumor immune responses prior to eventual cytolysis[19,20].

Nowadays, oncolytic virotherapy has shown its great potential in cancer therapy, and dozens of clinical trials are under way, some of which have been in phase Ⅲ. The rate of publication of manuscripts on oncolytic virotherapy has now surpassed that on viral cancer gene therapy[20]. In 2005, the Chinese State Food and Drug Administration approved the world's first oncolytic virus for treatment of cancer, an engineered human adenovirus (Oncorine; Shanghai Sunway Biotech, Shanghai, China) for treatment of head and neck carcinoma[21].

Target genes and efficacy

At present, cancer gene therapies are mainly based on two principles: gene augumentation and gene blockade. The former is introducing exogenous genetic materials (therapeutic genes) into cancer cells and let their expression products play a therapeutic role to prevent or reverse the growth of cancer cells, while the latter is inhibiting the excessive expression of intrinsic genes (target genes) in cancer cells. Therefore, the investigated genes of interest can be divided into same categories: therapeutic genes utilized for gene augumentation and target genes for gene blockade (Table 2). Since PC is one of the malignant diseases that have the most complicated genetics and pathogenesis, gene therapy of PC has encompassed almost all these categories mentioned above.

Tumor suppressor genes

$p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p14^{ARF}$, retinoblastoma protein (pRb) and p53: The *pRb* gene is a part of gene family that includes two other members, *p107* and *p130*, which collectively repress genes that regulates the G1 to S checkpoint of the cell cycle. Rb family proteins interact with transcription factor E2Fs, which induce the expression of genes needed for DNA synthesis. When bound at E2F-responsive promoters, Rb family proteins help to repress gene expression[22]. Rb proteins can be phosphorylated by cyclin dependent kinases 2, 4 and 6 (CDK2, 4 and 6), resulting in the release of E2F and gene expression[23].

The $p16^{INK4a}$ gene (*p16*), located on chromosome 9p21, is deleted in 85% of pancreatic adenocarcinomas[24]. It is the first member to be identified in the INK4 family of CDK inhibitors. The *p16* gene product is tight-binding and inhibitory protein for CDK4 to induce G1 arrest of the cell cycle[25], while $p21^{CIP1/WAF1}$ (*p21*) gene product acts as a downstream effector of p53 and mediates G1 cell cycle arrest by inhibiting CDK2. Loss of p21 activity has been observed in approximately 30%-60% of PC specimens[26].

The $p14^{ARF}$ (*p14*) gene, also located on chromosome 9p21, shares an exon with $p16^{INK4a}$ in different reading frames[27]. *Mdm2* (murine double minute) is a *p53*-inducible gene that normally acts to terminate the *p53*

Table 2 Target genes of gene therapy in pancreatic cancer

Strategy	Categories	Examples
Gene augumentation	Tumor suppressor genes	$p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p14^{ARF}$, Retinoblastoma Protein (pRb), *p53*, *p84 /Thoc1*, *p73*, *Smad4/DPC4*
	Drug sensitivity genes/ suicide genes	Herpes Simplex Virus Thymidine Kinase (*HSV-TK*), Cytosine Deaminase (CD), Nitroreductase (NTR), Cytochrome P450 (CYP)
	Anti-angiogenesis genes	Soluble VEGFR, Soluble FGFR, Endostatin, Thrombospondin-1, Angiostatin, Vasostatin, NK4, Matrix metalloproteinases inhibitors (MMIPs/TIMPs), Somatostatin receptors (SSTR)
	Immune related genes	MHC molecules, Co-stimulatory molecules (B7 family, ICAM-1, LFA-3), Inflammatory cytokines (IL-2, IL-12, GM-CSF, IFN-α, IFN-β, IFN-γ, TNF-α), Tumor antigen (CEA, MUC-1, *etc.*)
	Apoptosis related genes	TRAIL
Gene blockade/ antisense therapy	Oncogenes	K-ras, LSM1/CaSm, HER-2/EerB-2
	MDR	MDR1, MRP family, BCRP
	Proliferation related genes	VEGF, hTERT, COX-2

MDR: Multidrug resistance.

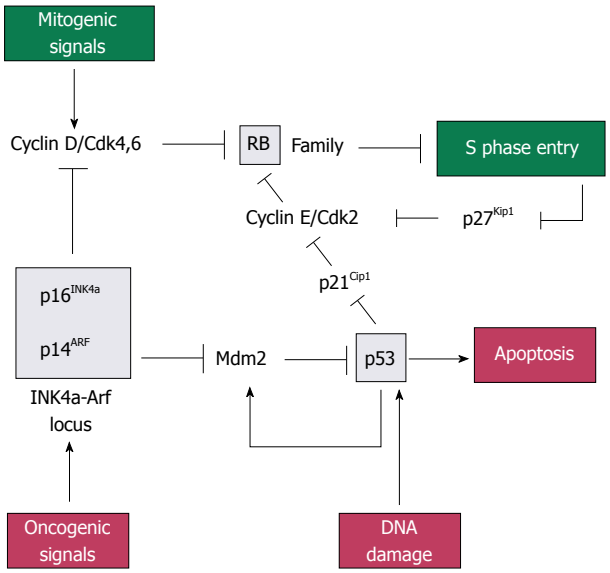

Figure 1 Cell cycle checkpoints. Mitogenic signals activate cyclin D-dependent kinases, which phosphorylate RB and RB family proteins (p107 and p130) to facilitate entry into S phase. The p16INK4a protein inhibits cyclin D/Cdk4, 6 to activate RB and prevent entry into S phase. The p14ARF protein inhibits Mdm2 to induce p53, leading either to p53-dependent apoptosis or to induction of the Cdk2 inhibitor p21Cip1. The p21Cip1 protein inhibits cyclin E/Cdk2 and induces RB-dependent cell cycle arrest.

response. The p14ARF protein inhibits *Mdm2* to induce *p53*, leading to p53-dependent apoptosis[22].

The *p53* gene, located on chromosome 17p, is inactivated by mutation in 70% of pancreatic adenocarcinomas. It can induce apoptosis or G1 cell cycle arrest *via* $p21^{CIP1/WAF1}$. It is normally maintained at a very low level by Mdm2, which targets p53 for ubiquitin-mediated degradation. Stress or mitogenic signals increase the level of $p14^{ARF}$, which in turn inhibits Mdm2 and lead to the stabilisation and activation of p53.

In short, pRb, p53, $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p14^{ARF}$ form part of a signaling network that monitors mitogenic signaling and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately (Figure 1)[22].

In research of PC therapy, both p16 and p21 have successfully been transduced into pancreatic cancer cell lines by means of adenoviral vectors, which results in growth inhibition and induction of apoptosis *in vitro*[25,28]. One study proved that p16-mediated cytotoxicity is tightly associated with the presence of functional pRb[29]. Liposome-mediated delivery of the $p14^{ARF}$ gene to pancreatic cancer cell lines was capable of resulting in the enhancement of their sensitivity to 5-Fu contrasting with cells devoid of $p14^{ARF}$ expression, which successfully inhibits PC cell proliferation[30].

Of all these tumor suppressor genes, *p53* is the most important and extensively studied one. The first gene therapy for the treatment of cancer in China in 2004 is a replication-defective adenovirus 5 expressing *p53* used for squamous cell carcinoma of the head and neck[21]. Transfer of the *p53* gene using an adenoviral vector also suppressed the growth of human pancreatic cancer cell lines *in vitro*[31]. Reintroduction of *p53* also increased cytotoxicity of gemcitabine *in vitro* and *in vivo*[32], and that of temozolomide *in vitro*[33]. In nude mouse model, intraperitoneal administration of the retroviral *p53* vector resulted in significant inhibition of the growth of primary pancreatic tumor and peritoneal deposits compared to controls[34]. What is more, the inhibitors of Mdm2 are also investigated as new agents for PC treatment, since they induced the growth inhibition through reactivation of the p53 pathway[35].

p84 /Thoc1: The *p84/Thoc1* gene encodes proteins that have similar death domains with other proteins involved in the regulation of apoptosis, which can bind to an amino terminal domain of the Rb1 protein, regulating transcriptional elongation and RNA processing. Its overexpression induces apoptosis of cancer cells. A recent study found that infection of pancreatic adenocarcinoma with adenovirus encoding *p53* and *p84/Thoc1* inhibited growth of cancer cells both *in vitro* and *in vivo* to a greater extent than treatment with either one alone[36].

p73: The *p73* gene, located on chromosome 1p36, is identified as a *p53* family member observed in 45.6% of pancreatic adenocarcinomas. It can induce cell cycle arrest and apoptosis in a p53 manner by binding to *p53* DNA target sites, and transactivates p53-responsive

genes[37]. One research found that an adenoviral vector encoding *p73* was capable of effectively killing several pancreatic cancer cell lines, including those that were completely resistant to p53-mediated apoptosis[38].

Smad4/DPC4: The *Smad4* gene, designated as tumor suppressor gene DPC4, is located on chromosome 18q21.1 and deleted in about 50% of pancreatic adenocarcinomas but only in about 10% or less of other cancers, which suggests that Smad4/ DPC4 may have a specific role in pancreatic tumorigenesis[39]. Smad4 is a member of the Smad family of transcription factors, which potentiates tumor growth, angiogenesis and invasion and is associated with poor prognosis[23]. Restoration of the *Smad4* gene using an adenoviral vector showed inhibition of pancreatic tumor growth in mice[40], while the same effect was seen *in vitro* through a retroviral vector pLXSN containing DPC4[41] .

Drug sensitivity genes/suicide genes

Drug sensitivity gene therapy, also known as gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy, attempts to selectively transduce tumor cells with a gene which, when express an enzyme, will convert a systemically administered nontoxic prodrug into a toxic metabolite.

A large number of enzyme-prodrug systems have been developed for suicide gene therapy in recent years. Examples of enzymes include viral thymidine kinase (TK), bacterial cytosine deaminase (CD), bacterial carboxypeptidase G2 (CPG2), purine nucleotide phosphorylase (PNP), thymidine phosphorylase (TP), nitroreductase (NR), D-amino-acid oxidase (DAAO), xanthine-guanine phosphoribosyl transferase (XGPRT), penicillin-G amidase (PGA), β-lactamase (β-L), multiple-drug activation enzyme (MDAE), β-galactosidase (β-Gal), horseradish peroxidase (HRP), deoxyribonucleotide kinase (DRNK), deoxycytidine kinase (dCK), carboxypeptidase A (CPA), β-glucuronidase (β-Glu), and cytochrome P450 (CYP)[42]. However, among these dazzling choices, the most classic paradigm in PC therapy is herpes simplex virus thymidine kinase (HSV-TK).

HSV-TK/ganciclovir

The *HSV-TK* gene codes for an enzyme that converts the nontoxic prodrug ganciclovir into monophosphorylated ganciclovir, which is subsequently further converted by cellular guanylate kinases to the triphosphorylated forms, blocking DNA synthesis and inducing cell death[43]. The therapeutic effect can also be amplified by a "bystander effect", which means *HSV-TK* transduced tumor cells are toxic to neighbouring unmodified tumor cells. The reason may be related to the uptake of toxic metabolites *via* intercellular communication paths such as gap junctions[44]. It is, in some extent, compensable for the low efficacy of gene transfer.

The HSK-TK delivered by retrovirus and adenovirus has been proved efficient in killing PC cells *in vitro* and *in vivo*[45,46]. The combination of adenovirus- and retrovirus-mediated delivery of HSV-TK appeared to be more effective in tumor reduction compared to either one alone *in vivo*[47]. Liposome mediated transfer of HSV-TK was able to cause regression of tumors in nude mice with peritoneal dissemination of PC[48]. However, there were some studies showing that retrovirally transduced HSV-TK had limited efficacy in PC cell lines both *in vitro* and *in vivo*[49,50]. The main reason of this controversial fact may be related to a poor efficiency of gene transfection *in vivo* and a limited bystander cell killing effect, so further study is required for its clinical application.

CD/5-fluorocytosine (5-FC)

CD is a bacterial enzyme that converts the prodrug 5-FC into the cytotoxic and radiosensitising agent 5-FU, which inhibits DNA replication and protein synthesis. Several studies have proved that the adenovirus carrying CD gene is efficient in inhibiting the growth of murine PC cell lines *in vitro* and *in vivo* when associated with 5-FC[51-53]. Furthermore, when combined with radiation, the adenoviral vector carrying a mutant bacterial *CD* gene (AdbCD-D314A) plus 5-FC significantly increased frequency of tumor regression and the persistence of tumor growth inhibition compared with either radiation or AdbCD-D314A/5-FC therapy alone[54].

There is another gene called *FUR1*, which encodes uracil phosphoribosyltransferase (UPRT), playing a role in CD/5-FC suicide therapy. Since the CD gene, also called *FCY1* gene, often demonstrates resistant to 5-FU, UPRT has an additional advantage as it catalyses the conversion of 5-FU into the toxic metabolite 5-fluorouridine-5'-monophosphate[55]. Combined treatment with 5-FU and E1B-55kDa-deleted adenovirus carrying the *UPRT* gene (AxE1AdB-*UPRT*) dramatically reduced the disseminated tumor burden in mice with peritoneal dissemination of AsPC-1 without causing toxicity in normal tissues[56]. One study showed that the *FCY1* gene alone was ineffective in the treatment of PC *in vitro* and plasmid vectors expressing chimera CD-UPRT (pRSV-CD-*UPRT*) only increased 5-FC sensitivity to some PC cell lines[57]. Another study demonstrated that adenoviral vectors carrying the CD: uracil phosphoribosyltransferase fusion gene (Ad-CD: *UPRT*) resulted in increased 5-FC-mediated cell killing, compared with Ad-CD[58]. Moreover, Ad-CD: UPRT/5-FC combined with monoclonal antibody TRA-8 produces an additive cytotoxic effect in cancer cells both *in vitro* and *in vivo*[58].

Nitroreductase/CB1954

The *Escherichia coli* enzyme nitroreductase (NTR) is able to convert the prodrug CB1954 (5-[aziridin-1-yl]- 2,4-dinitrobenzamide) to 2- and 4-hydroxylamino derivatives, which react with cellular thioesters to generate a potent alkylating agent capable of cross-linking DNA, inducing cell apoptosis eventually[59]. Retrovirus-mediated *NTR* gene delivery showed increased sensitivity up to 500-fold to CB1954 in PC cell lines *in vitro*, through associated by-

Table 3 Active and negative regulatory factors of angiogenesis

Stimulators	Inhibitors
Hypoxia	Angiostatin
Oncogenic proteins such as Ras	TSP-1
Inflammatory cytokines such as IL-8 and IL-6	Endostatin
FGF	Arrestin
TGF-β	Canstatin
HGF	MMPIs
PDGF	Somatostatin
G-CSF	Tumstatin
Angiogenin	VEGI
Leptin	Decoy receptors such as soluble VEGFR
Proliferin	Inflammatory cytokines such as IL-12

G-CSF: Granulocyte colony-stimulating factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; TGF-β: Transforming growth factor-β; FGF: Fibroblast growth factor; TSP-1: Thrombospondin-1; MMPIs: Matrix metalloproteinases inhibitors; VEGI: Vascular endothelial growth inhibitor.

stander effect[60]. In a nude mouse model with subcutaneous PC xenografts, retrovirus vectors expressing the *NTR* gene with administration of CB1954 resulted in tumor regression, growth delay and significantly increased median survival[61]. Another research of nude mouse xenograft model for disseminated peritoneal carcinomatosis with ascites (PC cell line SUIT2) showed that combination of replication-defective adenovirus vectors carrying the *NTR* gene (Ad-CMV-NTR) and *CB1954* almost doubled the median survival from 14 to 26 d[62]. The *NTR*/*CB1954* treatment has been tested in clinical trials of gastrointestinal and liver malignancies, but none for PC.

Cytochrome P450/cyclophosphamide

Cytochrome P450 enzyme converts the chemotherapeutic prodrugs cyclophosphamide (CPA) or iphosphamide (IPA) to toxic metabolites phosphoramide mustard, which is an alkylating agent able to form DNA cross-links in a cell cycle-independent manner. Since cytochrome P450 is predominantly produced in the liver and toxicity of the metabolite is rather systemic and not tumor-specific in human body, the transduction of the CYP gene into the tumor tissue shows its advantages to enhance tumor-specific toxicity.

In vitro study demonstrated that expression of CYP 2B1 enzymes (retrovirus-mediated transduction) led to an up to 13-fold increase in susceptibility to IPA in a range of PC cell lines (BxPC-3, MIA PaCa-2, Hs-766T, PaCa-44 and PANC-1)[63]. *In vivo*, retroviral CYP 2B1 transfer with CPA treatment highly sensitized PC cells NP-9, NP-18, and NP-31, and led to significant differences in tumor volume at the end of the treatment when compared with CPA alone[64]. Furthermore, in tumor-bearing mice model, intratumoral injection of encapsulated cells, which were genetically modified to express the CYP, was able to cause significant tumor reduction[65,66]. In addition, these encapsulated cells have also been used in phase Ⅰ/Ⅱ trials in patients with PC and have shown remarkable early success, with median survival doubled and 1-year survival improved by 3-fold[67].

ANTI-ANGIOGENESIS GENES

Tumor growth is dependent on angiogenesis, in which vascular endothelia growth factor (VEGF) plays a leading role. VEGF is a glycoprotein that has a huge impact on endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability. It is overexpressed in over 90% of PC and is associated with increased microvessel density, tumor progression and poor prognosis[68]. The VEGF receptor (VEGFR), which is a transmembrane receptor tyrosine kinase of the ErbB family, including VEGFR-1 (FMS-like tyrosine kinase-1, flt-1) and VEGFR-2 [fetal liver kinase-1 (flk-1) or kinase insert domain receptor (KDR)], is also overexpressed in the vasculature of tumors that express VEGF[69,70].

Mechanisms that lead to inappropriate activation of the VEGF pathway include receptor overexpression, activating mutations, overexpression of receptor ligands, and loss of their negative regulatory pathways. Both VEGF and VEGFR are, therefore, appealing targets for anti-angiogenesis therapy. Many molecular targeted agents and monoclonal antibody interfering with VEGF signal system have been developed for cancer therapy, such as Bevacizumab (a humanized antibody against VEGF), Sorafenib (a multi-targeted kinase inhibitor), Erlotinib (an inhibitor of EGFR, the only molecular targeted drug approved by the FDA in 2005 for PC), Axitinib (an inhibitor of both VEGFR and related tyrosine kinase receptors), and Aflibercept (a recombinant fusion protein that functions as a soluble decoy receptor and inhibits VEGF). However, these agents seem unlikely to confer sufficient benefit in the PC clinical trials and their cost-effectiveness has been questioned[71].

Compared to classic non-gene therapy, gene therapy represents a powerful tool for therapeutic intervention to angiogenesis in terms of specific targeting, cost-effectiveness and safety. In this new approach, the VEGF/VEGFR pathway is still the main hotspot. Stimulators and inhibitors that up-regulate and down-regulate VEGF signal pathways are all possible therapeutic targets in cancer treatment (Table 3)[72,73]. Therefore, strategies for anti-angiogenesis gene therapy can be divided into two categories: (1) delivery of genes encoding endogenous angiogenesis inhibitors or their receptors; and (2) blockage of the excessive angiogenesis genes encoding growth factors or growth factor receptors. The former is based on transfer of exogenous genes whereas the latter seeks to block excessive genes in tumor. Materials involved in these two are totally different.

Soluble VEGFR

Soluble forms of VEGFR-1 and VEGFR-2 are a kind of decoy receptor, which can inhibit VEGF dependent tumor angiogenesis, by binding to VEGF and acting as a dominant negative receptor[74]. Recombinant adenovirus-

es encoding soluble VEGFR-2 (Ad Flk1-Fc) and soluble VEGFR-1 (Ad sflt1) showed significant tumor inhibition when injected intravenously and directly into the tumors, respectively[75,76]. Crosslinked polyplex micelles modified by RGD (Arg-Gly-Asp) peptide ligands, a non-viral vector, carrying plasmid DNA expressing a soluble form of VEGFR-1 (sFlt-1), demonstrated significant inhibition of tumor growth *via* anti-angiogenic effect when systemically injected into pancreatic adenocarcinoma bearing mice[77]. A truncated dominant negative mutant of VEGFR-2, which binds to VEGF and decreases the angiogenic stimulus of VEGF, when delivered by replication-defective retroviruses, could also lead to inhibition of tumor growth in each of three human PC cell lines *in vivo*[78]. Similar results were also found in an *in vitro* study with herpes simplex virus (HSV) amplicon mediated delivery of a hypoxia-inducible soluble VEGFR-2 (sFlk-1)[79]. In an comparative research of the antitumor activity of anti-angiogenic proteins, recombinant adenoviruses encoding angiostatin, endostatin, neuropilin, and soluble forms of VEGFR (Flk1, Flt1) all resulted in inhibition of tumor growth through intravenous injection in murine models involving lung cancer, fibrosarcoma and PC, but soluble forms of VEGFR were significantly more effective (approximately 80% inhibition of preexisting tumor growth) than the others[80].

Soluble fibroblast growth-factor receptors (FGFRs)

FGFRs, encoded by four genes (*FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*), are involved in the regulation of organ development, cell proliferation and migration, angiogenesis and other processes. Recent studies have shown that FGFR-activating mutations and overexpression are closely associated with the development and progression of tumors in human[81]. Several monoclonal antibodies and small-molecule FGFR inhibitors have been developed, some of which have already entered early clinical development, such as AZD4547 (AstraZeneca), BGJ398 (Novartis), LY2874455 (Eli Lilly), GP369 (Aveo) and HuGAL-FR21 (Galaxy). Soluble forms of FGFR (sFGFR) had similar mechanism of action with sVEGFR. One study found that replication-defective adenoviral vectors carrying *sFGFR1* gene could effectively suppress tumor angiogenesis and enhance apoptosis among lung cancer cells and pancreatic cancer cells both *in vitro* and *in vivo*, especially in sVEGFR-resistant cancers. Furthermore, the combined usage of sVEGFR plus sFGFR1 produced an enhanced inhibitory effect compared to their individual effects[82].

Endostatin (ES), thrombospondin-1 (TSP-1), angiostatin (AS) and vasostatin

ES, AS, vasostatin and TSP-1 are the most important endogenous angiogenesis inhibitors that have been studied extensively. ES is a cleavage product from the C-terminal portion of collagen XVIII, which has been shown to inhibit endothelial cell migration and proliferation and induce apoptosis. The apoptotic effect of ES is associated with down-regulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, while inhibition of endothelial cell proliferation and migration is *via* interacting with several pathways, such as binding to integrin α5, associating with heparan sulfate proteoglycans on cell surface, and inhibiting VEGFR-2, PDGF, cyclin-D1 and metalloproteinases[83,84]. Recombinant human ES has been developed for cancer treatment and entered in clinical trials[85,86]. TSP-1 is a multifunctional extracellular matrix protein with pivotal roles in the regulation of vascular development and angiogenesis[87], while AS is a potent inhibitor of angiogenesis, selectively inhibiting endothelial cell proliferation and migration through binding a cell surface ATP synthase or inhibiting extracellular matrix (ECM)-stimulated plasminogen[72]. Vasostatin, the N-terminal domain of calreticulin, was also reported to have tumor suppressor and anti-angiogenic function *in vitro* and *in vivo*.

A double-regulated duplicative adenovirus expressing human ES (AdTPHre-hEndo) limited PC growth both *in vitro* and *in vivo*, and the inhibition was significantly higher than non-duplicative adenovirus vectors carrying the ES gene[88]. A eukaryotic expression vector pRC/CMV carrying the AS gene, when delivered into human PC cell line mediated by liposome *in vitro*, also notedly reduced the volume of tumors[89]. A replication deficient recombinant adenovirus encoding vasostatin (Ad-vasostatin) showed less neovascularisation and inhibited tumor growth *in vivo* and *in vitro*[90]. Same results were also discovered in a study with intratumoral delivery of recombinant AAV expressing vasostatin (rAAV-VAS)[91]. The Lister vaccine strain of vaccinia virus armed with the ES-AS fusion gene (*VVhEA*), which displayed high selectivity for cancer cells with effective infection of tumors after both intravenous and intratumoral administrations, showed a significant antitumor effect with evidence of inhibition of angiogenesis both *in vitro* and *in vivo*[92]. A recombinant AAV-mediated gene delivery of 3TSR (the antiangiogenic domain of TSP-1) or ES (rAAV-ES), *via* intratumoral injection, intramuscular injection or intrasplenic injection, also inhibited tumor growth by anti-angiogenic effect[87].

NK4

NK4, composed of the N-terminal hairpin and subsequent 4-kringle fragment of hepatocyte growth factor (HGF), is an HGF antagonist that acts as an angiogenesis inhibitor. NK4 strongly inhibits the infiltration, metastasis, and tumor growth of PC[93]. HGF is overexpressed in 61%-87% of PC cases[94] but frequently expressed in tumor-associated fibroblasts rather than PC cells. HGF promotes growth and enhances cell motility and extracellular matrix breakdown, leading to invasion and metastasis of cancer cells[95].

Strong antiangiogenic activity of an NK4-expressing adenvirus vector (Ad-NK) has been tested on PC cells in both *in vitro* and *in vivo* studies of subcutaneous and orthotopic transplantation, peritoneal dissemination, and liver metastasis[96]. Ad-NK potently inhibited the growth

and invasion of cancer cells in response to HGF[97]. Intrasplenic injection of Ad-NK4 suppressed the number and growth of hepatic metastases[98]. When injected into the peritumoral region combined with gemcitabine, Ad-NK4 completely suppressed peritoneal dissemination and liver metastases, leading to significantly increased survival, compared to Ad-NK or gemcitabine alone[96]. Intraperitoneal injection of Ad-NK4 could also suppress the development of peritoneal dissemination of PC in nude mice[99].

Matrix metalloproteinase (MMP) inhibitors

MMPs are a family of zinc-dependent proteolytic enzymes that degrade the extracellular matrix and play an important role in tumor progression, angiogenesis, immune evasion, invasion and metastasis[100]. Natural tissue inhibitors of metalloproteinases (TIMPs) regulate physiologic MMP activity and are implicated in malignancy. The imbalance between MMPs and TIMPs, which denotes overexpression of MMPs and reduced expression of TIMPs, is frequently found in PC[101]. Some synthetic MMPIs have been exploited for antitumor therapy, such as BAY 12-9566 and Marimastat, but showed limited survival benefit in clinical trials in spite of their effectiveness in animal models[102-104]. When undergoing gene transfection to overexpress *TIMP-1 in vitro*, human PC cells were less likely to implant, grow and migrate in nude mice and showed increased apoptosis, and decreased angiogenesis[105]. Adenoviral vectors encoding human TIMP1 or TIMP2 limited development of PC and led to prolonged survival *in vivo*[106].

Somatostatin (SST) receptors (SSTRs)

SST is a natural peptide hormone secreted in various parts of the human body, and participates in a wide variety of biological processes including neurotransmission and negative control of exocrine and endocrine secretions. Meanwhile, SST exerts a strong antiproliferative effect in normal as well as tumor cells by interacting with SSTRs, including apoptotic effects, growth factor inhibition, antiangiogenic and immuno-modulating activities[107,108]. SSTRs consist of five different G-protein coupled receptor subtypes (SSTR1-5), which are differently expressed in the various types of tumor. The antineoplastic activity of SST and its analogues depends on the receptor subtypes they are bound to. Among these receptor subtypes, SSTR-1 and SSTR-2 play a predominant role in mediating the anti-proliferative effect[109]. However, only *SSTR2* expression is significantly inactivated in 90% of PC cases and SSTR-2, therefore, occupies the majority of gene therapy studies of SSTRs[110,111].

When introduced into PC cell line PC-3 with lipofectamine, SSTR2 showed inhibition of *VEGF* and *MMP-2* expression *in vitro*[112]. A targeting adenoviral vector driven by MUC1-promoter expressing SSTR2 gene showed significant cell proliferation inhibition *in vitro*, though there was no AdMUC1-SSTR2-induced apoptosis[113]. Intratumoral transfer of SSTR2 using the synthetic vector linear polymers of ethylenimine (PEI), strongly inhibited tumor progression of pancreatic adenocarcinoma *in vivo*, while depleting SST by RNA interference completely reversed SSTR2's antitumoral effect on *VEGF* expression and tumor angiogenesis[107]. Another study demonstrated that SSTR2 restoration mediated by oncolytic adenovirus (ZD55-hSSTR2) alone had a minor antitumor effect, but antitumor efficacy can be enhanced with the combination of ZD55-TRAIL (TNF-related apoptosis-inducing ligand) *in vitro* and *in vivo*[114]. Gene transfer using SSTR1 also displayed growth inhibition of PC by inducing cell cycle arrest *in vitro* and *in vivo*[115]. What is more, cotransfection of *SSTR1* and *SSTR2* showed a synergistic inhibitory effect on tumor proliferation and rendered Panc-1 cells more responsive to an SST analogue[116].

IMMUNE RELATED GENES

Tumor cells generally have a low immunogenicity and are able to escape surveillance by the host. Cancer immunotherapy has been developed to overcome this immune tolerance, including active and passive immunity. Passive approach encompasses administration of cytokines, activated effector cells or specific monoclonal antibodies targeting tumor cells. Active immunotherapy involves stimulation of immune response to tumor-associated antigens (TAAs), *via* employing cancer vaccines. Meanwhile, gene therapy is helpful by transferring genes into tumor cells or immune cells to render them more immunogenic and more effective, respectively. This combination is also known as immunogene therapy (Figure 2)[117].

In pathway A, allogeneic or autologous tumor cell vaccines that are genetically modified to secrete cytokines or co-stimulatory molecules are designed to elicit systemic immune responses to attack tumor tissue. Though autologous tumor cell vaccines showed promising results in clinical trials, a number of technical problems were uncovered, including the requirement of labor-intensive procedures for production of an individualized vaccine and the difficulty of expanding primary human tumor cells to the high numbers required for vaccination[118]. Fortunately, allogeneic whole tumor cell vaccines had been also proved to successfully induce systemic tumor-specific immune responses without the need to be HLA compatible with the host, and became the major way in study of tumor vaccines[119]. In pathway C, immune cells, especially dendritic cells (DCs), are *ex vitro* cultured autologous cells that are genetically modified, stimulated by specific antigens or activated by multiple cytokines to bypass the dysfunction of endogenous immune cells, restore immune surveillance, induce cancer regression or stabilization or delay and prevent its recurrence.

Host immune responses against a tumor antigen include cellular and/or humoral immune responses, starting with the processing of tumor antigens by antigen presenting cells (APCs) and recognition by T lymphocytes. T-cell activation requires not only the interaction between

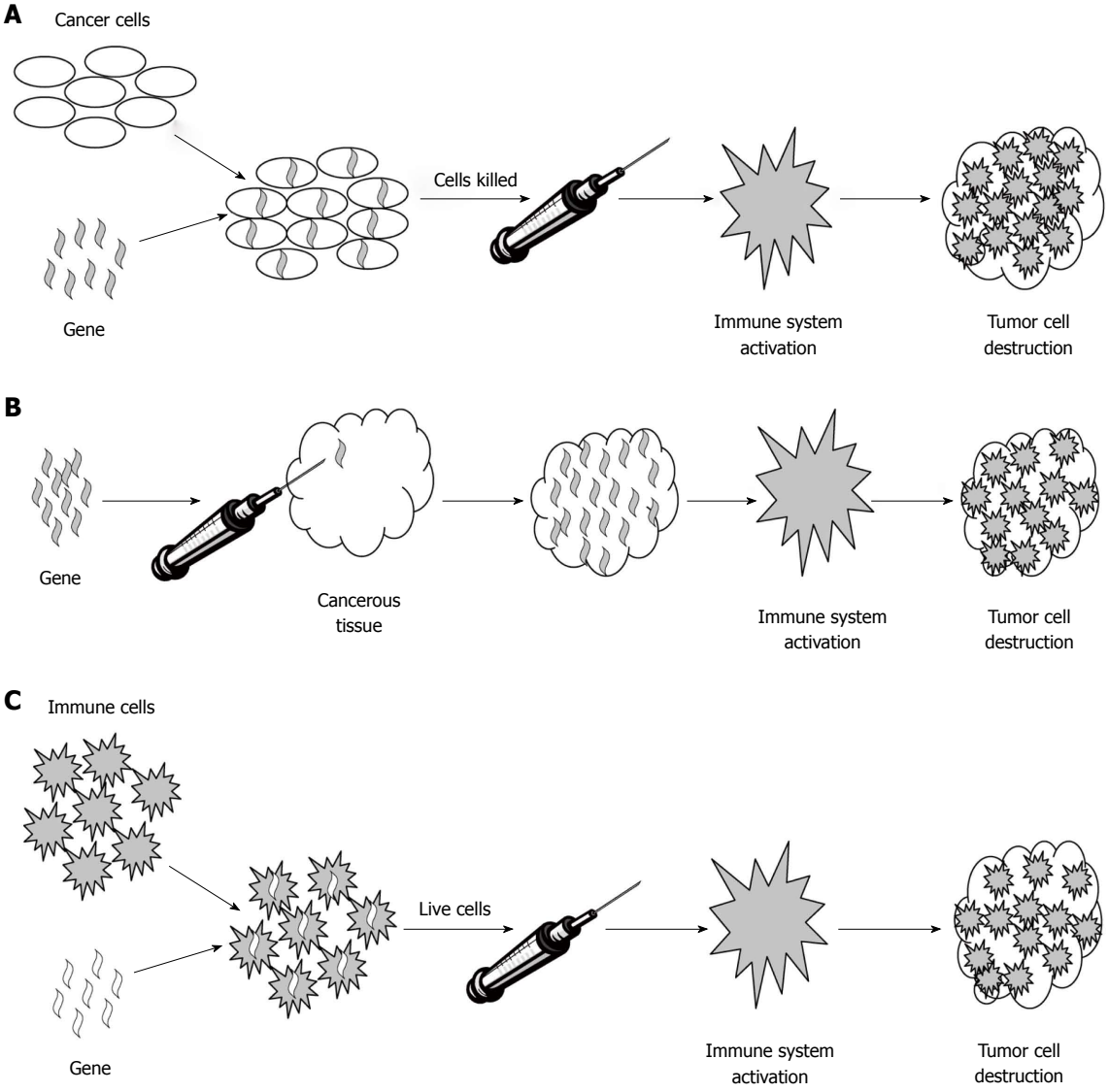

Figure 2 Schematic diagram of immunogene therapy. A: Immunogene therapy with altered cancer cells, which are harvested from patients (autologous cells) or from established cancer cell lines (allogeneic) and then cultured *in vitro* and inactivated to yield a vaccine; B: Immunogene therapy with *in vivo* gene transfer; C: Immunogene therapy using altered immune cells.

major histocompatibility complex (MHC) antigens bearing a specific peptide and the T-cell receptors, but also non-antigen-specific co-stimulatory activation by interaction of molecules expressed on the T-cells and APCs. Mechanisms of tumor immune tolerance include: underexpression of MHC antigens, loss of co-stimulatory molecule expression, alteration of tumor antigens, and secretion of immunosuppressive factors. Aiming at these mechanisms, at least four kinds of genes can be utilized in immunogene therapy: (1) major histocompatibility complex (MHC) antigens/human leukocyte antigens (HLAs), (2) co-stimulatory molecule genes, (3) tumor antigen genes; and (4) inflammatory cytokine genes.

MHC molecules and co-stimulatory molecules

Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Therefore, one approach to induce tumor-specific immune responses is to genetically modify tumor cells to express MHC molecules and co-stimulatory molecules on their cell surface. This kind of modification makes tumor cells to function as professional APCs and enhance their ability to directly stimulate T cells. In addition, this way is also appropriate for manipulation of DC cancer vaccines[120].

Loss or reduced expression of MHC-class-I molecules in many cancer cells have been found in mice and humans and have been identified as an important form of immune evasion. Despite that a large number of potential cancer antigens were discovered, clinical trials of immunization were disappointing due to the loss of *MHC-I* expression in tumors[121]. In animal models, *in vivo* gene transfer of foreign MHC-class-I *H-2K* gene into tumors successfully induced a cytotoxic T-cell response and attenuated tumor growth and caused complete tumor regression in murine models[122,123]. Transfection of

tumor cells with syngeneic MHC-class-Ⅱ or allogeneic MHC-class- Ⅰ genes improved tumor-specific immunity in the autologous host[124]. Therefore, combining MHC molecules with other tumor antigens could emerge as an attractive approach in cancer immunotherapy. No correlative research in PC has been reported yet.

In studies of costimulatory molecules, B7 family is one of the most important members and is constitutively expressed by most APCs, as the ligand for two receptors expressed on T cells, CD28 and CTLA-4. In animal studies, transfection of B7.1 into some tumors resulted in tumor rejection and generated systemic immunity against wild-type tumor challenges *via* stimulating CD8+ T cells[125]. Cotransfection of B7 with MHC-Ⅱ molecules was capable of inducing potent systemic immunity *via* both CD4+ and CD8+ T cells pathways[126]. A combinatory vaccine regimen (PANVAC-VF) composed of vaccinia virus and fowlpox virus expressing tumor-associated antigens (CEA and MUC1) and costimulatory molecules [B7.1, intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-3 (LFA-3)], when administered by subcutaneous injection with adjuvant GM-CSF, showed a significantly great therapeutic effect both in animal models and in clinical trials with advanced PC patients[127,128].

Inflammatory cytokines

Numerous cytokines have been studied in *in vivo* gene transfer. Human PC cells that underwent retrovirus-mediated gene transfer of IL-2, IL-4, IL-6, IL-27 and granulocyte macrophage-colony stimulating factor (GM-CSF) showed significant retardation and even regression when inoculated into BALB/c nude mice[129,130]. *In vivo* gene delivery of IL-1β, IL-24, IFN-α, IFN-β and IFN-γ by different viral vectors have been proved to lead to significant tumor growth inhibition in many PC models[131-135]. In addition, combined application of cytokine transfection and traditional chemotherapy, and combining immune genes with either tumor suppressor genes or suicide genes, are also common strategies in cancer immunogen therapy. For instance, combination of intratumoral human TNF-α gene delivery with gemcitabine produced marked delays in the growth of human pancreatic xenograft tumors relative to either agent alone *in vivo*[136,137]. Combination of IFN-α gene delivery with 5-FU had similar results as well[137]. An *in vitro* study of combining INF-β gene tranfection with gemcitabine also showed tumor growth inhibition[138]. In a phase Ⅰ / Ⅱ trial, intratumoral gene delivery of replication-deficient adenovirus encoding TNF-α, combined with standard chemoradiation, showed promising clinical outcome with dose-limiting effect and toxicity[139].

In studies of tumor cell based vaccines, cytokine genes have been also introduced into tumor cells to render them tumorigenicity and immunogenicity. One study that directly compared and contrasted effects of different cytokines in murine tumor models demonstrated that the tumors transduced with GM-CSF produced the greatest degree of systemic immunity relative to irradiated non-transduced tumor cells[140]. In a phase Ⅰ trial of allogeneic GM-CSF-secreting cancer vaccines in 14 patients with pancreatic adenocarcinoma, no local or systemic dose-limiting toxicities were observed and three subjects who received the highest two dose levels showed increased disease-free survival time (more than 2 years)[141]. In a comparative clinical trial of GM-CSF-secreting cancer vaccines alone and combined with cyclophosphamide in patients with metastatic PC, minimal treatment-related toxicity was found and cyclophosphamide cohort exhibited longer progression-free survival and overall survival[142]. Another clinical trial of ipilimumab (anti-CTLA-4) in combination with allogeneic PC cells transfected with a *GM-CSF* gene also showed prolonged disease stabilization in previously treated advanced PC[143].

Tumor antigens

Clinical studies of immunotherapy in cancer have focused on five classes of tumor antigens: (1) tumor-specific antigens (MAGE-1, NY-ESO-1, TRAG-3, PSA); (2) mutated oncogene products (p53, K-ras, HER2, BCR/abl, WT-1); (3) reactivated embryonic gene products (CEA, AFP); (4) self-antigens overexpressed in tumors (MUC1, survivin); and (5) oncogenic virus antigens (EBV, HPV, HBV)[120,144]. Many antigen-specific vaccines that urge the host immune system to recognize the primary tumor have been developed, including recombinant viral and bacterial vaccines that encode tumor antigens, peptide-or protein-based vaccines that mixed with adjuvants, DNA-based vaccines expressing tumor antigens, and antigen-pulsed DC vaccines[120]. Up to now, at least 75 antigens were identified that had many of the study-defined characteristics of an ideal candidate antigen for cancer therapy. Many of these antigens are the focus of targeted therapy in clinical trials in cancer (Table 4)[144-149].

It is encouraging that many of the results of PC vaccine trials verified the safety and immunogenicity of these vaccines. In a mutated K-ras peptide vaccine clinical trial with 48 patients in PC, when combined with adjuvant GM-CSF, more than 50% of patients demonstrated a tumor-specific immune response and significantly improved median overall survival *vs* their non-responding counterparts[150]. A multi-institution double-blinded placebo-controlled trial of gastrin peptide vaccine in 154 patients with advanced-stage PC demonstrated a nearly 2-fold increase in the median overall survival in the treatment compared to the placebo group[151]. A phase Ⅰ / Ⅱ clinical trial of telomerase peptide vaccines also demonstrated prolonged survival in immune responders *vs* non-responders[152]. In a multi-institutional, open-label, dose-finding, phase Ⅱ trial of Algenpantucel-L, 70 patients with resected PC were administered by two different doses of the vaccine in combination with gemcitabine and 5-fluorouracil. Of the PC patients who received a higher dose of vaccine, 96% survived for at least one year in comparison to the historical control of 69%, showing a statistically significant difference[153].

Table 4 Antigenic sources used in pancreatic cancer vaccine trials

Peptide or protein vaccines	Mutant K-ras peptide
	WT1 peptide
	CAP1-6D
	G17DT
	HLA-A*0201 restricted VEGF receptor-1 and -2 peptides
	HLA-A24 restricted survivin-2B80-88 peptide (AYACNTSTL)
	Autologous heat shock protein HSPPC-96
	TELOVAC
Whole cell vaccines or dendritic cell vaccines	α-Gal transferase transfected allogeneic tumor cells (including Algenpantucel-L)
	GVAX
	MUC-1 pulsed autologous dendritic cells
Viral or bacterial vaccines	PANVAC-VF-MUC-1, CEA, and TRICOM transfected virus
	Mesothelin (CRS-207) transfected live-attenuated Listeria
DNA vaccines	VXMO1

VXMO1: VEGF receptor-2 DNA vaccine; GVAX: GM-CSF transfected allogeneic tumor cells; TELOVAC: Telomerase peptide GV1001; CAP1-6D: CEA peptide; WT1: Wilms tumor 1; G17DT: Gastrin peptide.

Although encouraging, results from single-agent immunotherapy clinical trials have been underwhelming. As more and more tumor antigens are identified, more specific and potent vaccines will be developed. The ideal vaccine will target multiple antigens that are crucial to the growth and progression of tumors. Perhaps combinatorial therapeutic approach, which includes chemotherapy, radiation, surgery, and immunotherapy, will result in even greater survival benefits for patients with PC[13].

APOPTOSIS RELATED GENES

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL or Apo-2 ligand (Apo-2L) is a type Ⅱ transmembrane protein belonging to the TNF superfamily and serves as an effective anticancer agent due to its cancer cell specificity and potent antitumor activity. TRAIL interacts with death receptor 4 (DR4) and DR5, which form the death-inducing signal complex (DISC) by binding to Fas associated death domain (FADD), thereby activates caspase-8 and results in activation of downstream caspases-3, -6, and -7, and apoptosis induction[154]. Recombinant human TRAIL has been developed as a novel anticancer agent and is being clinically evaluated for the treatment of both solid tumors and hematological malignancies, such as colorectal, melanoma, lung, ovarian cancers and non-Hodgkins lymphoma[155]. However, its use *in vivo* is limited by a short half-life in plasma due to a rapid clearance by the kidney.

Gene delivery of TRAIL into tumors may overcome this limitation. An adenoviral vector expressing TRAIL driven by a human telomerase reverse transcriptase (hTERT) promoter showed specific antitumor efficacy in PC cell lines *in vitro* and significantly suppressed tumor growth *in vivo*[156]. Systemic administration of this vector in combination with chemotherapy (gemcitabine) exhibited a synergistic effect in the induction of apoptosis[157]. TRAIL-engineered pancreas-derived mesenchymal stem cells (MSCs) were able to induce PC cell apoptosis *in vitro*[158]. Another study of adipose-derived MSCs that were transduced with the TRAIL gene showed that, when injected intravenously or subcutaneously into mice, these MSCs localized into tumors and mediated apoptosis without significant apparent toxicities to normal tissues[159]. These studies implied that MSCs may serve as a stable source of TRAIL delivery in PC therapy.

ONCOGENES

K-ras

Ras is the most common oncogene detected in human cancers. It comprises 3 families, H-ras, K-ras, and N-ras. Of these, the K-ras family is responsible for almost all of the PC mutations, with mutations in the other families occurring rarely. Studies suggested that K-ras, which is located on chromosome 12p13, is mutated in up to 95% of PC cases[16,160]. The *K-ras* encodes membrane-bound GTP-binding proteins, which can be activated by signaling partners to regulate many cellular functions, including cell growth, proliferation, and differentiation. Mutations of *K-ras* result in malfunction of GTPase and participate in the initiation or early phase of pancreatic tumorigenesis[71].

To blockade the Ras signaling pathway, cancer vaccines that stimulate immunity against mutant Ras proteins and antisense therapy that blocks the translation of mutant Ras gene are two common strategies. Antisense therapy involves the use of oligonucleotides, ribozymes and siRNAs. Retroviral delivery of K-ras siRNA to human tumor cells induced loss of expression of the K-ras gene, leading to loss of anchorage-independent growth and tumorigenicity *in vitro*[161]. Another study of K-ras siRNA delivered by electroporation demonstrated significant tumor growth inhibition both *in vitro* and *in vivo*. When K-ras siRNA is combined with gemcitabine, survival rate was significantly prolonged and the mean tumor volume was dramatically reduced when compared with single agents[162].

K-ras antisense oligodeoxynucleotide (K-ras-ASODN) was identified to successfully inhibit *K-ras* expression[163] and suppress the growth and invasiveness of PC cell lines *in vitro*[164]. In peritoneal dissemination models of PC, intraperitoneal injection of adenovirus expressing antisense K-ras RNA significantly suppressed the peritoneal growth with no significant systemic toxicity[165]. K-ras ASODN combined with type I insulin-like growth factor receptor (IGF-IR) antisense oligodeoxynucleotide (IGF-IR-ASODN) showed a significant inhibitory effect on tumor growth and induced apoptosis *in vitro* and *in vivo*, compared with each agent alone[166]. In a phase Ⅱ trial of pa-

tients with locally advanced and metastatic PC, ISIS 2503, a phosphorothioate oligonucleotide antisense inhibitor of human H-ras mRNA, showed a response rate of 10.4% and a median survival of 6.6 mo in combination with gemcitabine, which is promising but of unclear benefit[167]. Initial enthusiasm for this approach is currently diminishing following the failures of antisense inhibitors such as ISIS 3521 (a protein kinase C-alpha antisense oligonucleotide) and oblimersen (a bcl-2 antisense oligonucleotide) in lung cancer and melanoma, respectively.

LSM1

The cancer-associated Sm-like (CaSm) oncogene LSM1 has been reported to be overexpressed in 87% of PC cases[168]. An adenovirus expressing CaSm antisense RNA (Ad-alpha CaSm) reduced endogenous CaSm mRNA expression *in vitro*, and a single intratumoural dose of Ad-alpha CaSm inhibited tumor growth and extended survival time in an *in vivo* SCID mouse model of human PC. The antitumor effect was further enhanced by gemcitabine[168]. In a metastatic tumor model, systemic administration of Ad-alpha CaSm resulted in a significant decrease in the number of hepatic metastases and increased survival time through both direct and bystander effects[169].

HER-2/ErbB-2

Human epidermal growth factor receptor 2 (HER-2) is a transmembrane receptor tyrosine kinase of the ErbB family. It participates in the EGFR signaling pathway and has roles in cell proliferation, survival, motility, invasion and adhesion. Blocking of overexpressed HER-2 oncogene was able to improve survival in breast and gastroesophageal cancers. Some studies found that HER-2 amplification occurs in 2% of pancreatic ductal adenocarcinomas (PDACs) but has distinct features with implications for clinical practice, which represents an attractive target for anti-HER2 therapies[170]. However, clinical trials of anti-HER2 antibodies such as trastuzumab showed no benefits compared with standard chemotherapy and did not recommend further evaluation of anti-HER2 treatment in patients with metastatic PC[171].

MULTIDRUG RESISTANCE GENES

Drug resistance is a major cause of treatment failure in cancer chemotherapy. One of the important mechanisms of tumor multidrug resistance is increased drug efflux and decreased accumulation of drugs in the cell. Efflux transporters of the ATP-binding cassette (ABC) family such as ABCB1 (multidrug resistance 1, MDR1), the ABCC (multidrug resistance-associated protein, MRP) family, and ABCG2 (breast cancer resistance protein, BCRP) have been identified as major determinants of chemoresistance in tumor cells[172].

MDR1 is a classic paradigm in the ABC family and has been analyzed in detail in PC. Its product, P-glycoprotein, is a membrane protein that functions as an ATP-dependent exporter of drugs from cells. Some studies demonstrated a high rate (73.2%) of *MDR1* expression in PC[173,174], and other studies reported that MDR1 is associated with sensitivity to gemcitabine[175]. The MRP family consists of 9 members (MRP1-9) and is involved in exporting a variety of endogenous substrates as well as organic anions of xenobiotics, conferring cells resistance to cytotoxic and antiviral drugs[176]. It has been shown that the expression of MRP3 and MRP5 mRNAs was upregulated in PC and MRP3 was even correlated with tumor grade[176]. BCRP was first derived from a resistant breast cancer cell line, but is present in a wide range of human solid tumors, including PC[177]. One study proved that BCRP expression was frequent (73.1%) in PC, and high BCRP expression was a significant prognostic factor for early tumor recurrence and poor survival[178]. Conversely, a study demonstrated low BCRP mRNA levels in both normal pancreatic tissues and PC[176].

In fields of anti-*MDR* gene therapy for PC, some studies have showed that siRNAs against *MDR1* could specifically inhibit *MDR1* expression at the mRNA and protein levels and decreased resistance against daunorubicin in PC cell lines *in vitro*[179]. Similar results were found in another study of a hammerhead ribozyme against MDR1 mRNA[180]. However, *in vivo* studies and clinical trials are still scarce in this field and there is much room for advancement to validate their clinical applicability.

Another important approach of making use of the *MDR* gene is transducing the *MDR* gene into hematopoietic stem cells to strengthen the host's resistance to myelosuppression caused by chemotherapeutic drugs. An advantage of this approach is that it permits higher doses of chemotherapy without severe adverse effects, thus providing a better chance to achieve remission. *In vitro*, lentiviral or retroviral vectors encoding MGMT (P140K) and MDR1 or MRP1 resulted in significant survival advantage of human hematopoietic stem cells when undergoing intensification chemotherapy, compared with untransduced cells or either single vector alone[181-183]. In mouse models, *MDR1* gene modified hematopoietic cells also showed chemoprotective effect from various anticancer drugs[184,185]. So far, several clinical trials of MDR1 transfected autologous hematopoietic stem cell transplantation have been approved for the treatment of patients with breast cancer, ovarian cancer or leukemia. However, since the expression rate of the *MDR1* gene in PC is very high, this system may not be useful for treating this cancer.

PROLIFERATION RELATED GENES

VEGF

As mentioned above, the VEGF signal pathway plays a leading role in tumor angiogenesis. Therefore, knockdown of VEGF gene expression is a promising way in anti-angiogenesis therapy in PC. Materials in this approach include antisense oligonucleotides, ribozymes and siRNAs.

One study of antisense oligodeoxynucleotide of

VEGF-C showed that it decreased the expression levels of VEGF-C and inhibited lymphangiogenesis in nude mice with orthotopically xenografted human PC, but had no significant effect on angiogenesis[186]. This result was verified in another study with short hairpin RNA (shRNA) targeting *VEGF-C*, and the VEGF-C shRNA significantly inhibited cell proliferation and tumor growth *in vivo*[187]. On the other hand, antisense oligonucleotide of VEGF was proved to significantly decrease neoangiogenesis and vascular permeability in orthotopic xenograft models; furthermore, it reduced tumor growth and metastasis and improved survival[188]. Systemic or intratumoral injection of VEGF specific siRNAs also led to the significant reduction in the subcutaneous tumor growth through down-regulating VEGF expression and decreasing microvascular density[189,190]. A study with hammerhead ribozymes against VEGF gene transcripts showed inhibition of tumor growth and liver metastasis of a PC cell line *in vivo*[191]. However, no clinical trial in this approach has been reported in PC.

Human telomerase reverse transcriptase

Telomere is a region of repetitive nucleotide sequences at each end of a chromatid, which plays a critical role in maintaining chromosome stability. Telomere is shortened progressively during normal cell division. When its length becomes critically short, it triggers replicative senescence or apoptosis. Telomerase is a ribonucleoprotein polymerase that maintains the length of telomere. Human telomerase complex consists of telomerase reverse transcriptase (hTERT), telomerase RNA (hTR or TERC), telomerase associated protein-1 (TEP-1), hsp90 and p23, of which the RNA subunit and hTERT constitute the core of telomerase. It has been tested that hTERT is the limiting component of telomerase and its expression levels parallel to those of telomerase activity[192,193]. Generally, telomerase activity is detectable only in germ line cells and certain stem cells but is repressed in somatic cells. Upregulated telomerase activity is associated with promotion of tumorigenesis, neoplastic growth and metastasis of human cancer[194,195]. In fact, approximately 85% of human cancers exhibit reactivation of telomerase activity, which is even as high as 92%-95% in PC[196,197]. Recently, one new viewpoint declares that hTERT is also implicated in DNA repair and regulation of the expression of genes that control cell proliferation, which promotes tumorigenesis independent on the stability of telomere[198]. In a nutshell, hTERT is an important proliferation-related factor and can serve as a tumor marker and a prognostic indicator.

In vitro, hTERT antisense oligonucleotide (hTERT-ASODN) could down-regulate expression of hTERT mRNA and increase cell apoptosis rate in a concentration- and time-dependent manner in PC cell lines[199,200]. Therefore, consecutive transfections were performed in order to inhibit telomerase activity and result in a continuous reduction in cell viability[201]. Cell cycle analysis indicated that the cells were mainly arrested at the G0/G1 phase with the treatment of hTERT-ASODN. In addition, hTERT ASODN synergized with gemcitabine to exert an anti-proliferation effect[200]. Similar results were demonstrated in another study of hTERT-siRNA transfection in pancreatic cancer cell line Capan-2, and the inhibitory effect was associated with the downregulation of *Bcl-2* and cyclooxygenase-2 (*COX-2*)[202]. Hammerhead ribozyme targeting hTR was also found to depress telomerase activity, and ribozyme targeting hTERT mRNA showed stronger inhibition. Since the level of hTERT mRNA expression is less than that of hTR expression in cancer cells, hTERT might be a more useful therapeutic target[203]. An hTERT peptide vaccine GV1001 was tested in a phase Ⅰ/Ⅱ study of 48 patients with unresectable PC. The patients received intradermal injection in combination with GM-CSF. In the end, 24 of 38 evaluable patients demonstrated immune responses with the highest percentage (75%) in the intermediate dose group. Approximately 8.6 mo of mean survival for this group was significantly longer and one-year survival rate was 25%[152]. These promising results have led to commencement of another phase Ⅲ trial of GV1001.

COX-2

COX-2 is a key enzyme of the metabolic process of arachidonic acid and an early response protein that is induced rapidly by growth factors, tumor promoters, oncogenes, and carcinogens. It plays an important role in tumorigenesis and angiogenesis. It has been shown that COX-2 mRNA and protein expression was highly upregulated in up to 90% of PC cases but was undetectable in nontumorous pancreatic tissue[204,205], and this indicated that COX-2 may be a potential target for treatment of PC. One study showed that COX-2 siRNA transfection could inhibit cell proliferation, induce cell apoptosis and regulate cell cycle of a PC cell line *in vitro* and decrease its tumorigenicity when inoculated subcutaneously into nude mice[206]. Nevertheless, inhibition of COX-2 is more applied in anti-inflammation treatment as nonsteroidal anti-inflammatory drugs (selective inhibitors of COX-2) than gene therapy.

Clinical research of gene therapy

Up to July 2013, 1970 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide. Of these, 1264 (64.2%) have been made for treatment of cancers, including lung, gynecological, skin, urological, neurological and gastrointestinal tumors, as well as hematological malignancies and pediatric tumors. In this part, we summarized the finished and ongoing clinical trials of PC gene therapy worldwide (Tables 5 and 6) and the administration routes they employed (Figure 3). Meanwhile, since a variety of cancers have common characteristics, such as mutation of tumor suppressor genes or oncogenes and overexpression of tumor antigens, one therapeutic transgene can be effective for different tumors. In fact, many clinical trials are indicated for several cancers simultaneously, like all solid tumors

Table 5 Closed clinical trials of gene therapy in pancreatic cancer

Trial ID and investigator	Phase	Clinical indication	Patients (*n*)	Transgenes	Vectors and target cells	Administration route	Combined therapy	Results
DE-0009 Löhr *et al*[67]	Ⅰ / Ⅱ	Inoperable PC	14	Cytochrome p450	Naked/plasmid DNA transfected allogeneic human 293 embryonic kidney cells	Inject into the tumor vasculature *via* supraselective angiography	In combination with low-dose ifosfamide	4 patients showed tumor regression, the other ten individuals remained stable. Median survival was doubled compared with historic controls. 1-yr survival rate was three times better
DE-0024 Pecher *et al*[207]	Ⅰ / Ⅱ	Advanced breast cancer, PC and gallbladder carcinoma	10 (2 PC)	MUC-1	Naked/plasmid DNA transfected autologus dendritic cells	Subcutaneous injection	None	9 patients showed signs of progression. Only one remained stable for 3 mo until she was transferred to another therapy. 3 of 10 patients developed vaccine-specific delayed-type hypersensitivity reaction (DTH). 4 of 10 patients showed increased mucinspecific INF-gamma-secreting CD8+ T cells
DE-0063 Kubuschok *et al*[208]	Ⅰ	PC	3 healthy donors and 1 PC patient	Mutated ras oncoprotein	EB virus transformed autologous lymphoblastoid cells	Subcutaneous injection	None	All the subjects showed strong vaccine-induced muRas-specific cytotoxic T lymphocytes
DE-0083 Niethammer *et al*[209]	Ⅰ	Advanced PC	45	VEGFR-2	Naked/plasmid DNA (oral DNA vaccine)	Oral administration	In combination with gemcitabine	Not reported
ES-0004 Mazzolini *et al*[210]	Ⅰ	Liver cancer, PC, colorectal cancer	17 (3 PC)	Interleukin-12 (IL-12)	Adenovirus transfected autologous dendritic cells	Intratumoral injection	None	Treatment was well tolerated. 11 of 17 were assessable for response. A partial response was observed in 1 case with PC. Stable disease was observed in 2 patients and progression in 8 patients
FR-0018 Gilly *et al*[211]	Ⅰ / Ⅱ	Unresectable digestive cancer	6 (3 PC)	Interleukin-2 (IL-2)	Adenovirus	Intratumoral injection	None	Good safety. But final results of tumor responses were not reported
US-0853 Le *et al*[212]	Ⅰ	Ovarian cancer, PC, lung cancer, mesothelioma	28	Mesothelin	Listeria monocytogenes	Intravenous injection	1: Live attenuated Listeria vaccine (n = 9); 2: live attenuated mesothein expressing Listeria vaccine (n = 17)	In arm 2, Listeriolysin O and mesothelin-specific T-cell responses were seen and 37% of subjects lived ⩾ 15 mo
US-0700 Galanis *et al*[213]	Ⅰ	Gemcitabine-refractory, metastatic PC	12	Cyclin G1	Retrovirus	Intravenous injection	None	Good safety. But there was no evidence of anti-tumor activity
Kaufman *et al*[214]	Ⅰ	Advanced PC	10	CEA, MUC-1, and TRICOM (including B7.1, ICAM-1, LFA-3)	Poxvirus	Subcutaneous injection	In combination with GM-CSF	Antigen-specific T-cell responses in 5 of 8 evaluated patients. 15.1 mo of median survival in responders *vs* 3.9 mo in non-responders. Overall median survival is 6.3 mo
Jaffee *et al*[141]	Ⅰ	Resected PC	14	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	3 of 14 patients developed DTH, and 3 patients had increased disease-free survival time (at least 25 mo after diagnosis)
US-0475 Lutz *et al*[215]	Ⅱ	Resected PC	60	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	The median disease-free survival is 17.3 mo with median survival of 24.8 mo. Induction of CD8+ mesothelin-specific T cells correlated with disease-free survival
Laheru *et al*[142]	A pilot study	Advanced PC	50	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	A: vaccine alone (n = 20) B: vaccine plus cyclophosphamide (n = 30)	Cohort B showed enhanced mesothelin-specific T-cell responses. Median survival values in cohort A and cohort B were 2.3 and 4.3 mo
Le *et al*[143]	Ⅰ b	Advanced PC	30	CM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	1: Ipilimumab alone (n = 15); 2: Ipilimumab plus vaccine (n = 15)	Median overall survival was 3.6 mo for arm 1 and 5.7 mo for arm 2. Mesothelin-specific T cells responses were associated with increased disease-free survival in arm 2

Table 6 Ongoing clinical trials of gene therapy in pancreatic cancer

Phase	Transgenes
I	Oncolytic adenovirus and oncolytic herpesvirus[1]
	Somatostatin receptor 2 (sst2), Deoxycitidine kinase :: uridylmonophosphate kinase (dck::umk)
	Somatostatin receptor 2 (sst2)
	Mesothelin-scFv with signaling domains comprised of TCR , CD28, and 4-1BB (CD137) cDNA
	GM-CSF
	PANVAC (CEA, MUC-1, and TRICOM)
	Cytosine deaminase, Herpes simplex virus thymidine kinase (HSV-TK)
	Cyclin G1
	p53
	BikDD (DOTAP-cholesterol mediated gene transfection)
	Mutated Ras
I / II	Cytochrome p450
	AEG 35156: antisense oligonucleotide to X-linked inhibitor of apoptosis protein (XIAP)
	Herpes simplex virus thymidine kinase (HSV-TK)
	Diphtheria Toxin A Chain (DTA) gene with H19 promoter
	Alpha-(1,3) galactosyltransferase
II	CEA, MUC-1, and PANVAC
	Mutated Ras
	Alpha-(1,3) galactosyltransferase
III	Alpha-(1,3) galactosyltransferase
	PANVAC (CEA, MUC-1, and TRICOM)[2]

[1]Clinical trials of oncolytic viruses were completed or are ongoing in Spain and Japan, and their details were not clear; [2]This clinical trial has closed, but details and results have not been published yet.

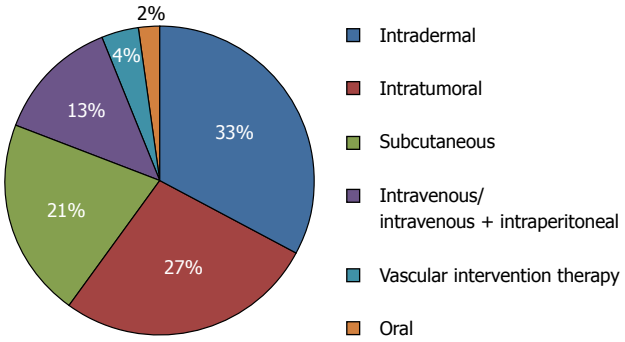

Figure 3 Clinical trials regarding administration routes of gene therapy in pancreatic cancer.

or advanced tumors. Therefore, here we also collected such kind of trials and obtained a list of them (Table 7). It is worth noting that our analysis is mainly based on the records in The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide website (http://www.wiley.co.uk/genmed/clinical), so trials in some countries (*e.g.*, Japan) are not available.

Table 7 Clinical trials of unspecific tumor gene therapy that may involve pancreatic cancer

Phase	Clinical indication	Transgenes
II	Adenocarcinoma	CEA, TCRzeta and CD28
I	Cachexia	GHRH
I	CEA- or HER-2-expressing malignancies	HER-2, CEA
I	CEA-expressing malignancies	CEA
I / II	CEA-expressing malignancies	CAP-1 peptide from CEA
I / II	CEA-expressing malignancies	T cell receptor alpha and beta chains cDNA
I	CEA-expressing malignancies	CEA, B7.1, ICAM-1, LFA-3, GM-CSF
I	CEA-expressing malignancies	Anti-CEA-SFv-Zeta T cell receptor
I	CEA-expressing malignancies	B7.1 (CD80)
II	Advanced cancer with overexpression of p53	Anti-p53 T cell receptor
I	MUC-1- expressing tumors	MUC-1, IL-2
I	Advanced cancer	Cytochrome P450
I	Advanced cancer	Endostatin
I	Advanced cancer	Heat shock protein 70
I	Advanced cancer	T cell receptor alpha and beta chain
I	Advanced cancer	Bifunctional shRNA specific for stathmin 1 oncoprotein
I / II	Advanced cancer	GM-CSF
I	Advanced cancer	IL-12
I / II	Advanced cancer	CYP1B1
I	Advanced cancer	GM-CSF, CD154 (CD40-ligand)
I / II	Advanced cancer	IL-2
I	Advanced cancer	p53
I	Advanced cancer	AMEP
I	Advanced cancer	B7.1 (CD80) ,ICAM-1, LFA-3
I / II	Advanced cancer	Cytosine deaminase
I / II	Advanced cancer	CEA
I	Solid tumors	Tumor antigen
I	Solid tumors	Interferon-gamma
I	Solid tumors	TNF
I	Solid tumors	Brachyury oncoprotein
I	Solid tumors	Human telomerase reverse transcriptase
I / II	Solid tumors	Oncolytic virus (no transgene)
I	Solid tumors	Retinoblastoma 94
I	Solid tumors	p53
I	Solid tumors	O6-methylguanine DNA methyltransferase (MGMT)
I	Solid tumors	GM-CSF
I	Solid tumors	GM-CSF, bi-shRNA-furin
I	Solid tumors	GM-CSF, TGF-beta 2 antisense
I	Solid tumors	GM-CSF, humanized *Escherichia coli* beta-galactosidase

CEA: Carcinoembryonic antigen; IL-2: Interleukin-2; AMEP: Antiangiogenic metargidin peptide; CYP1B1: Cytochrome P450 isoenzyme 1B1; TNF: Tumor necrosis factor; hTERT: Human telomerase reverse transcriptase; GHRH: Human growth hormone releasing hormone.

Conclusions and prospects

Over the past decades, plenty of experimental works and clinical trials have demonstrated the safety and efficacy of cancer gene therapy. Strategies encompass tumor suppressor, suicide, anti-angiogenesis and immune related genes, as well as activated oncogenes and multidrug resistance genes. Proliferation and apoptosis related signal pathways are also possible targets. However, among these therapeutic targets, only a small part were tested in clinical settings and proved to be effective, and even for them, outcomes were far from curative and treatments have to be combined with standard chemo- or radio-therapy for maximum benefits.

In this field, tumor suppressor gene *p53* and mutant

oncogene *K-ras* are two members that have been most deeply studied in all kinds of cancers and have yielded encouraging results *in vitro* and in animal models. Unfortunately, these findings have not been translated to improve outcomes in clinical trials. In suicide strategy, HSV-TK, CD and cytochrome p450 have been proved to be efficacious. A closed clinical trial of cytochrome p450 showed exciting results when combined with ifosfamide, and more trials are ongoing. As to anti-angiogenesis approach, VEGFR is of course the best choice, clinical trials of peptide vaccine derived from VEGFR-2 and DNA vaccine targeting VEGFR-2 did show benefits, but as a monotherapy it is not a "magic bullet" for all tumor patients since anti-angiogenesis is far more effective in preventing tumor growth than causing regression of established tumors, thus this method may be more suitable for patients with minimal residual disease. Nowadays immunotherapy has become the mainstream direction of studies in searching new cancer treatment regimens, and the cooperation of gene therapy and immunotherapy has created even more inspiring achievements, such as adoptive transfer of genetically modified T-cells or DCs, DNA vaccines (CEA, MUC-1, B7.1, ICAM-1, LFA-3), and direct transfer of cytokine genes (IL-2, IL-12, GM-CSF, INF-gamma). Of course, combining different targets or combination of gene therapy and traditional chemoradiotherapy may lead to improved efficacy of gene therapy in PC.

Considering that low efficiency of gene transfer is the main obstacle in application and popularization of gene therapy, the selection of high-efficient and tumor-targeted vectors should be emphasized. In general, viral vectors offer higher transduction efficiency and long-term gene expression and this is why more than two-thirds of clinical trials reported employ viral vectors. Recently, oncolytic viruses that infect and replicate selectively in cancer cells have become the hotspot in this field and show a bright future. Oncolytic viruses produced from initially infected cancer cells can spread to surrounding cancer tissue and distant tissue, thus intratumoural injections could be efficient, even for the treatment of disseminated tumors. Systemic administration of oncolytic viruses has also shown good safety and this is of great interest particularly in PC, since it is difficult to inject viruses into primary lesions directly in PC patients.

Gene therapy of cancer is not yet applied in routine clinical practice, but good safety records and validated clinical benefits make it a good candidate for all clinical settings including neo-adjuvant, adjuvant and palliative treatment. Despite these promising therapeutic strategies, greatest advance in the treatment of PC may still come from improved early detection and diagnosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (15): Laparoscopic resection of gastrointestinal

Laparoscopic liver resection: Wedge resections to living donor hepatectomy, are we heading in the right direction?

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Abstract

Despite inception over 15 years ago and over 3000 completed procedures, laparoscopic liver resection has remained mainly in the domain of selected centers and enthusiasts. Requirement of extensive open liver resection (OLR) experience, in-depth understanding of anatomy and considerable laparoscopic technical expertise may have delayed wide application. However healthy scepticism of its actual benefits and presence of a potential publication bias; concern about its safety and technical learning curve, are probably equally responsible. Given that a large proportion of our work, at least in transplantation is still OLR, we have attempted to provide an entirely unbiased, mature opinion of its pros and cons in the current invited review. We have divided this review into two sections as we believe they merit separate attention on technical and ethical grounds. The first part deals with laparoscopic liver resection (LLR) in patients who present with benign or malignant liver pathology, wherein we have discussed its overall outcomes; its feasibility based on type of pathology and type of resection and included a small section on application of LLR in special scenarios like cirrhosis. The second part deals with the laparoscopic living donor hepatectomy (LDH) experience to date, including its potential impact on transplantation in general. Donor safety, graft outcomes after LDH and criterion to select ideal donors for LLR are discussed. Within each section we have provided practical points to improve safety in LLR and attempted to reach reasonable recommendations on the utilization of LLR for units that wish to develop such a service.

Key words: Liver; Laparoscopic; Transplantation; Resection; Living donor; Minimally invasive; Technique; Hepatocellular; Hepatectomy; Cirrhosis

Core tip: Given that a liver resection is ideally suited for a laparoscopic approach (no anastomosis, very large incision in open approach) there is increasing interest in the technique worldwide. However actual experience is limited to a few centres, and guidelines remain inadequate. This article summarises the current available evidence on the significant aspects of the technique individually, to guide clinicians considering developing such a practice.

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INTRODUCTION

Since the first non-anatomical laparoscopic liver resection (LLR) in the early 1990s, and the first anatomic LLR in 1996[1], there have been over 3000 cases performed laparoscopically clearly demonstrating its feasibility. However nearly 80% of these have been selected (*i.e.*, benign lesions, sited in the anterior aspect of the liver) or relatively minor resections(single or bisegment)with larger resections being confined to a handful of enthusiasts worldwide so far. The combined requirements of long experience with open liver resection (OLR); an in-depth understanding of liver anatomy; considerable technical laparoscopic expertise and a healthy scepticism regards its actual benefits are probably equally responsible for this slow uptake.

A potential danger with assessment of a new technique is the influence of institutional and publication biases, in that negative reports have not made it to press, as noted during the Louisville meeting[2]. Moreover all or most of these procedures are performed in centres with an ardent interest in minimally invasive liver resection (MILR) and it is difficult not to let some "subjectiveness" into reports. On the other hand there is a learning curve with all procedures and overemphasis on individual mishaps could delay application of a good innovation as with experience complication rates decrease, akin to common bile duct injury in laparoscopic cholecystectomy. Given that our work, especially in liver transplantation is mostly open liver surgery, we have attempted to be entirely unbiased in the current invited review. We have divided this review into two sections as we believe they merit separate attention on both ethical and technical grounds. The first part deals with MILR for liver pathology (benign and malignant) in patients wherein we have discussed this approach on its overall outcomes, outcomes based on type of resection, its feasibility based on type of pathology and finally MILR in special scenarios like cirrhosis. At the end of each section we have tried to provide a reasonable conclusion. The second part deals with the laparoscopic living donor hepatectomy (LDH) experience including its impact on transplantation in general. As discussed previously, we agree MILR has the potential to convert previously unfit or unwilling donors into donation, but equally, negative outcomes could result in a significant impact on programmes such as ours, where over 85% of patients depend on such donation, for transplantation.

MILR IN PATIENTS, FOR LIVER DISEASE

Laparoscopic liver resection: outcomes, feasibilty, benefits and morbidity

Outcomes: Needless to say, success of a technique is often decided by its overall outcome and the most decisive basic outcome measure is mortality. In the world review of LLR by Nguyen *et al*[3] in 2009, 9 patients of 2804 died showing an overall mortality of 0.3%. Mortality rate in LLR is consistently low in most original series and remains between1and 1.8%[4,5]. In fact in some studies, mortality figures are superior in the LLR compared to OLR[6]. A meta-analysis of laparoscopic and open hepatic resection done by Mirnezami *et al*[7] analyzed the short and long-term outcomes of 1678 patients. Thirty-day mortality occurred in 0.6% of patients undergoing LLR (4 out of 717) compared with 1% of patients undergoing OLR (10 out of 961). Even in LLR for malignancy, there was no significant difference in survival or disease recurrence when compared to OLR whether it was an anatomical or a non-anatomical resection[8]. On the contrary, no study to date has shown LLR to be associated with greater mortality, when compared to the open approach[9]. On study of the mortality that has occurred, none were intra-operative and the most common cause for post-operative mortality remained liver failure, as with OLR[3]. However mortality was related to "haemorrhage" from major blood vessels in some series[5,10]. Median blood loss is significantly more for right-sided LLR than for the left sided liver surgery but whether it contributes to mortality is still unclear[11]. Of course, mortality rates are significantly higher (0.3% *vs* 5.8%) in the cirrhotic versus non-cirrhotic resection group, but this would be true for OLR too[4,12].

Feasibility: Feasibility is the next outcome measure that requires study, and intra-operative conversion to an open approach is a surrogate marker for feasibility. Of course, feasibility also depends on type of resection and site of lesion, but this will be discussed elsewhere. Conversion rates are variable but most large series quote figures around 2%-4%[4,5]. Conversion from laparoscopy to open laparotomy and from laparoscopy to hand-assisted approach occur in 4.1% and 0.7% of reported cases according to Nguyen's world review[3]. In the series reported by Kazaryan *et al*[5], overall conversion rate was 3.4%. Conversions to laparotomy were because of intra-abdominal adhesions (2 cases) and haemorrhage (3 cases). Repeated resections after open liver resection are associated with a significantly higher rate of conversion to laparotomy but in most series, the conversion is due to intra-operative bleeding[11]. In a recent series by Troisi *et al*[13] the conversion rate was higher at 6.4%, and on univariate analysis, they identified "major" hepatectomy and resections involving posterior-superior segments as prognostic factors for conversion whereas multivariate analysis identified the latter as an independent risk factor. The Mirnezami *et al*[7] meta-analysis found the conversion-to-open rate for all laparoscopic procedures was 7% (50 out of 717). However, it is important to stress that conversion from a pure laparoscopic method to hand assisted or laparotomy should and must not be considered a complication. The Louisville consensus conference summarised the opti-

mal approach well, by stating that all efforts should be made to control bleeding laparoscopically and conversion should be done in a staged manner starting from a hand assisted technique and finally laparotomy, as a significant blood loss can occur during conversion[2]. They noted that patient safety and lack of progress were the primary indications worldwide for conversion, which is how it should be.

Benefits: Given above literature findings, LLR is feasible and safe in comparison with OLR. But does the technique actually deliver on the two most quoted reasons for its perusal- enhanced recovery and maintenance of abdominal wall integrity? Indeed, the most significant benefit of LLRs besides others is "lengths of hospital stay", an easily measured but debatable surrogate for enhanced recovery. Most studies have consistently demonstrated a significantly lower hospital stay as compared to the open approach. Buell et all reported a mean hospital stay of 2.9 d as compared to 5.4 d in the open group[4]. Kazaryan *et al*[5] 2010 further elaborated that the length of hospital stay does not depend on LLRs done for either benign or malignant lesions, or anatomical and non-anatomical resections. For all kinds of resections their mean hospital stay was 3 d. Zhou *et al*[14] pooled analysis of the 6 studies of LLRs for colorectal liver metastasis(CRLM) showed that hospital stay was shorter in the laparoscopic group(WMD: -3.54, 95%CI: -5.12-1.96; $P < 0.001$) in spite of significant heterogeneity in their patient cohort. The overall shorter hospital stay in LLRs is not merely 'faster discharges' but a by-product of an earlier return of bowel activity and lesser requirement of analgesia[14,15]. The Louisville position is consistent with such studies and further states that the benefits of shorter hospital stay extend to both major and minor LLRs but added that these results may be dependent on the level of experience of surgeons in both advanced laparoscopy and liver surgery[2]. Moreover given that financial considerations dictate some of our clinical decisions, cost-effectiveness of LLR has been looked into. Most mature series agree that LLR is indeed cost-effective, with minor increase in cost of consumables in theatre being offset by immense decrease in the cost of hospital stay, regardless of type of resection[6,16]. This opinion was endorsed by the consensus conference[2].

Morbidity: Despite comparable mortality, overwhelming morbidity clearly needs to be ruled out. However complications in LLRs are comparable in most reports and significantly lower than the open approach in many. Post-operative morbidity of LLR varies between 10% and15% and is significantly lower than open liver resection(about 25%)[17-19]. Several studies including Rowe *et al*[6] in their study found that LLRs had significantly decreased intra-operative blood loss (287 mL *vs* 473 mL, $P = 0.03$) and post-operative complications (6% *vs* 42%, $P = 0.03$)[7]. In the series by Buell, the overall complications in LLR were about 16% *vs* 22% in the open group[4]. In fact, patients appear to be more likely to have complications or morbidity in the open resection group than in the laparoscopic group for both the simpler antero-lateral and more complex postero-superior resection subgroups[20]. Furthermore we know that complication rates generally tend to decrease, as experience and exposure in LLRs increase[21]. Predictably LLR is also associated with a higher complication rate when resections involve posterior/superior segment of liver, and tumors > 5 cm are resected[21,22]. Most complications due to LLR appear to belong to Clavien grade1 and 3 subgroups when they do occur[13].

Intra-operative bleeding during LLR is a traumatic experience for most surgeons. A combination of large calibre, thin walled, high flow hepatic and portal veins, with variable anatomy, hidden within liver parenchyma makes parenchymal transection dangerous in inexperienced hands and is one of the reasons why LLR has not been taken up with the same enthusiasm as other laparoscopic procedures. But with the right technique and experience, perhaps surprisingly, several reports confirm that blood loss is significantly lower in LLR when compared to open[14-16,23-25]. Mirnezami *et al*[7] meta-analysis showed a median blood loss of 320 mL for LLR (range 122-620 mL) and 483 mL for OLR (range 214-895 mL). This is potentially due to higher magnification of structures viewed through high definition cameras, use of vessel sealing energy devices /endoscopic staplers, control of resection by laparoscopic ultrasonography and the automatic, conscious efforts by laparoscopic surgeons to clip/control even small vessels before their division at laparoscopic transection[20,26]. We have found laparoscopic curved vascular clamps and energy devices like the LigaSure®Covidian Surgical solutions essential components in the operating set.

Post-operative bile leakage was observed in around 1%-2.7% and most of them were managed by percutaneous drainage with or without bile duct stenting[3,16,27]. Once again, better visibility from the on-screen magnification presumably accounts for this low rate of bile leaks. As with other surgery, morbidity associated with LLR can be due to general complications like chest and urinary tract infections, deep vein thrombosis, paralytic ileus, or pyrexia's of unknown aetiology. But due to the faster mobilisation and improved respiratory function, in reality, incidences appear to be low. Awareness must exist of unusual procedure related complications like iatrogenic port insertion injury, rupture of the splenic capsule whilst performing a Pringle manoeuvre *etc.,* Gas (CO_2) embolism (GE) is a rare complication found during LLR and risk of GE is higher if dissection involves a major hepatic vein, long transaction plane, or a longer operative time. It is likely that a combination of low central venous pressure (CVP; which creates a greater suction effect), and high intra-abdominal gas pressure (IAP; which clearly encourages entry of gas into an open "sucking" vein) is responsible for GE. Hence a low IAP and use of an "abdominal lift" system has been used to minimize the

Table 1 Comparison of mortality, morbidity and conversion rates in some of the larger laparoscopic liver resection publications

Ref.	Year	Cases (*n*)	Malignant lesions (%)	Major hepatectomies (%)	Mortality (%)	Morbidity (%)	Conversion (%)
Descottes *et al*[92]	2003	87	0	3 (3.4)	-	4 (4.6)	9 (10)
Mala *et al*[93]	2005	53	89	-	-	8 (16)	3 (5.6)
Kaneko *et al*[51]	2005	52	NR	NR	-	5 (10)	1 (2)
Vibert *et al*[27]	2006	89	73	38 (43)	1 (1.1)	31 (34.8)	12 (13)
Cai *et al*[94]	2006	62	32	2 (3.2)	-	2 (3.2)	2 (3.2)
Koffron *et al*[16]	2007	273	37	96 (35)	-	NR	-
Chen *et al*[95]	2008	116	100	4 (3.4)	-	7 (6)	6 (5.2)
Buell *et al*[4]	2008	253	42	62 (42.5)	4 (1.6)	41 (16)	6 (2.4)
Bryant *et al*[32]	2009	166	60	31 (18.7)	-	25 (15.1)	16 (9.6)
Dagher *et al*[23]	2009	22	22	22	0	14	9
Yoon *et al*[39]	2010	69	-	21 (20)	-	5	5
Martin *et al*[25]	2010	90	90	90	0	23	4
Abu Hilal *et al*[24]	2011	36	-	36	0	14	11

risk[28]. On the other hand, there are experimental studies in animals that showed GE occurred irrespective of the CVP-IAP gradient, suggesting other mechanisms might contribute to GE occurrences[29]. Further studies are required to clearly identify the role of CVP and IAP, but it is important to note that CO2 embolism is not as dangerous as air embolism, given its higher/faster solubility (in blood)[30]. However patients who based on ECHO/capnography have clearly experienced CO2 embolism, have gone on to have no harmful sequelae[31,32].

Conclusion: In conclusion therefore, when compared with open liver resection, LLR can provide comparable mortality, shorter hospital stay, improved morbidity and better cosmesis, cost effectively (Table 1). Clearly, feasibility of most resections is now proven with very low conversion rates. Enhanced recovery with short hospital stays are real and not merely faster discharges. Decreased bile leak rates and consistently lower blood loss in many large series are significant advantages. LLR must be carried out under as low IAP as possible, with modern energy devices to complete transection, particularly laparoscopic CUSA® (Cavitron ultrasonic surgical aspirator, Valleylab, United States) and laparoscopic ultrasound.

Laparoscopic liver resection: type of resection

Historically anterolateral segments (segments 2, 3, 5, 6), were considered more amenable for LLR, and initial attempts at LLR were limited to non-anatomical resections of such segments. In most of the early reports, lesions in posterior-superior segments were even considered a relative contra-indication for LLR[33,34]. But innovative techniques and increasing experience has led to a gradual progression towards left sectionectomies, right and left hepatectomies and finally posterior segment resections. Indeed almost all types of liver resections are currently being performed routinely, with investigators claiming equivalent or superior results with the laparoscopic as compared to the open approach, although most major hepatectomies still utilize a hand assisted or hybrid technique than pure laparoscopy[4,23,24]. It has even been shown by several operators that even segment one (caudate), can be resected in isolation or as part of another major hepatectomy[4,35]. Nevertheless, despite passage of over 15 years since the first LLR, the 2009 world review of LLR shows that an overwhelming majority of resections are still either segmentectomies (45%) or anatomic left lateral sectionectomies (20%) with anatomical hemihepatectomies constituting a lesser proportion of LLR [right (9%) and left (7%)][3]. We analyzed more than a 1000 LLRs in five recent, major case series[4,5,16,32,36]. Of these patients, a vast majority (nearly 80%) of resections were again segmentectomies, left lateral sectionectomies or non-anatomical resections. In our opinion, this persistent trend accentuates the point that in most major hepatectomies, surgeons still prefer OLR, an approach that might be related to the fact that the type of resection correlates directly with morbidity and operating times[13,34].

Given enormous differences between "minor" and "major" LLR in terms of expertise required, morbidity and intra-operative technique, we decided to review them separately after an arbitrary division into: (1) minor LLR which includes left lateral sectionectomy, segmentectomy, anterior bisegementectomy and non-anatomical resections of anterolateral segments; and (2) major LLR which includes right and left hepatectomies, extended right and left liver resections. At this point it must be added that many authors consider a left hepatectomy (although a "hemihepatectomy") to be imminently suited for LLR, without the increase in morbidity noted with right or postero-superior LLR[37-39]. Moreover as most left liver specimens are < 450 g in weight, extraction through small incisions is feasible and an added attraction.

In a review of a recent series by Troisi *et al*[13] major hepatectomy and resections involving posterior-superior segments were identified as prognostic factors for conversion on univariate analysis, whereas multivariate analysis identified the latter as an independent risk factor. Recently Yoon *et al*[39] compared outcome after LLR of HCC situated in the anterolateral segment versus those in the postero-superior segment and found that the postero-superior patients had longer operative time ($P = 0.001$) longer hospital stay ($P = 0.039$), higher rate of open conversion ($P = 0.05$) and greater blood loss ($P = 0.068$), but with no

significant difference in post-operative complications or cancer recurrence. Other reports also reconfirm that major hepatectomies were associated with higher blood loss (mean 313 mL *vs* 128 mL; $P = 0.05$), longer hospital stay, recovery and operative time[34]. Yet despite the above findings, even on comparison of major hepatectomies alone, surgical outcomes were better in LLR in terms of blood loss, complication rates, and length of hospital stay compared to OLR[23-25,40]. However, it is important to note that some segmental or sectional resections such as a posterior sectionectomy performed laparoscopically can be more technically demanding than a hemi-hepatectomy, given that these are often performed without inflow control at the hilum, occur in an awkward plane and surprisingly, involves more extensive areas of parenchymal transection than an anatomical right hepatectomy.

Single incision liver resections [Laparoendoscopic single-site surgery (LESS)] are now being performed for smaller liver lesions with equivalent or superior results. Tan *et al*[41] used in 7 patients successfully. Five left lateral sectionectomies, one segment 3, and one segment 5 resection were performed. Similar results were reported by Aikawa *et al*[42] who performed 8 single site Lap liver resections. However discussion of this technique is beyond the scope of this report.

Conclusion: Therefore in our opinion, there is enough evidence to suggest that for certain resections such as segmentectomies, wedge resections, left lateral sectionectomies and even left hemi-hepatectomies when compared with open liver resection, LLR appears to provide comparable mortality, shorter hospital stay, improved morbidity including decreased blood loss and better cosmesis, cost effectively. It can be suggested that for these procedures a laparoscopic approach should be the standard of care at centres where surgeons are experienced in both liver surgery and advanced laparoscopy. However, it is yet to be conclusively established whether these benefits are extendible to major/postero-superior segment laparoscopic hepatectomies as data is still evolving and patient numbers small. It is essential to recognize requirement of considerable experience in at least 100 major OLR before attempting minor LLR and at least 50 minor LLR before major LLR (opinion).

Laparoscopic liver resection: type of pathology

LLR for benign liver disease: Removal of concern about 'tumour clearance' makes LLR attractive in benign liver lesions and indeed many surgical units started with LLR being limited to benign conditions. On an overview of the current literature, approximately 25% of LLR in benign disease were for cystic lesions, followed by 21% for haemangioma, 18% for focal nodular hyperplasia, 7% for adenomas and 5% for hepatico-lithiasis. Understandably most of these patients treated for benign disease were younger (mean age 48 years) and female (M/F 1/8.5) compared to overall LLR cohorts[32,43,44]. LLR for other benign lesions such as localized intra-hepatic duct dilatation, and Caroli's disease limited to segment Ⅱ and Ⅲ have also been reported with comparable results, in term of operative time, blood loss and mean hospital stay[45-47]. Although difficult to accurately estimate, one worries that introduction of a less invasive procedure might lead to more benign lesions are being managed surgically. At the risk of stating the obvious we wish to emphasize that "minimal invasive" is not equal to "non-invasive" and indication for surgery must not change[48].

LLR in malignant liver disease: On review of reports published over the last 7 years, we found that approximately 58% of LLR were done for malignancy, most of which were for Hepatocellular carcinoma (HCC; 57%)or CRLM. Customary concerns when utilising laparoscopic surgery for malignancy, includes anticipated difficulties in intra-operative tumour localization with deep seated tumours, achievement of a safe tumour free margin and port site seeding. However in a meta-analysis of LLR for malignancy, Mirnezami *et al*[7] did not find any significant difference in the size of the resection margin with LLR when compared with OLR (OR = -0.356; 95%CI:-1.061-0.349, $P = 0.318$) and similarly in the world review by Nguyen *et al*[49] negative surgical margins were achieved in 82%-100% of reported series, which is clearly comparable to other OLR series. A single center series published in 2011, reported comparable mid-term survival between pure laparoscopic and open right hepatectomy for liver metastases[3,24].

Widespread use of intra-operative ultrasound has more or less removed concerns about the lack of tactile feedback as an aid to tumour localisation[28]. Concerns about a higher risk of peritoneal tumour seeding and subsequent recurrence due to positive pressure pneumoperitonium and use of liver transection devices which could disseminate tumour cells in the spray created during device oscillation have been raised. We scrutinized studies reporting on comparative oncological outcome data for LLR and OLR. Data from these numerous studies many of which are reporting on mature cohorts with at least medium term follow-up, indicates an equivalent recurrence rate between LLR and OLR, indeed proving that above mentioned concerns are unfounded[8,50-53]. Moreover in none of these reports do specifically either port site metastasis or tumour seeding mentioned as a major concern even with medium to long term follow-up after LLR for malignancy. Hence it can be concluded that LLR presents no greater risk for malignant liver tumours than classic OLR. A number of other authors also agree that the benefits of almost all laparoscopy techniques can be extended to malignant lesions without compromising oncological principles[54]. Given that the bulk of LLR for malignancy are for two causes, we reviewed below, outcomes in LLR specifically for HCC and CRLM.

LLR for CRC metastasis: Liver resection is now considered standard treatment for CRLM. Kazaryan *et al*[5] reported a 10-year Norwegian single-centre experience in

Table 2 Some of the major laparoscopic liver resection for colorectal cancer series

Ref.	Year	*n*	Operative time(min)	Mortality	Blood loss (mL)	Conversion (%)	Hospital stay (d)
Rau *et al*[96]	1998	17	183	0	457	6	7.8
Shimada *et al*[97]	2001	17	325	0	400	0	12
Mala *et al*[98]	2002	13	187	0	600	0	4
Kaneko *et al*[51]	2005	30	182	0	350	3	14.9

Table 3 Some of the major laparoscopic liver resection for hepatocellular cancer series

Ref.	Year	PTS (*n*)	Cirrhosis present (%)	Tumor size (cm)	Morbidity (%)	Mortality (%)	OS (1 yr/5 yr, %)	RFS (1 yr/5 yr, %)
Dagher *et al*[36]	2010	163	73.6	3.6	22	1.2	92.6/64.9	77.5/32.2
Aldrighetti *et al*[99]	2010	16	56.2	4 + 2.2	25	0	-	-
Nitta *et al*[100]	2010	15	80	4.5	11.6	0	-	-
Yoon *et al*[39]	2010	69	55	3.1	21.7	0	90.4 (3 yr)	60.4 (3 yr)
Cherqui *et al*[101]	2009	37	74	-	34	0	-/72	-/44
Bryant *et al*[32]	2009	64	28	3.5	15.1	0	70 (3 yr)/65 (5 yr)	46 (3 yr)/34 (5 yr)
Chen *et al*[95]	2008	116	100	2.1	6	0	90/61	-
Buell *et al*[4]	2008	36	56	4.6	16	1.6	90/80	23 (2 yr)
Kaneko *et al*[51]	2005	30	100	3	10	0	97/61	87/31
Teramoto *et al*[102]	2005	11	67	2	26	0	100/80	75/40

OS: Overall survival; RFS: Recurrence-free survival.

96 patients undergoing LLR for CRLM(median tumour size 3.0 cm), and described a R0 resection rate of 94%, and 5-year overall survival of 46%, comparable to large open resection series. As most CRLM would have received hepatotoxic pre-operative chemotherapy, a specific concern with LLR in CRLM was intra-operative bleeding (due to liver parenchymal fragility) and post-operative liver failure and compromised liver regeneration after a partial hepatectomy as shown by Sturesson *et al*[55] Zhou *et al*[14] pooled analysis of the 6 studies of LLRs for colorectal metastasis showed that hospital stay was shorter in the laparoscopic group($P < 0.001$), once again with lower blood loss and no greater incidence of post-operative liver failure, despite significant heterogeneity in the analysis cohort. In fact recently synchronous resections of primary colorectal tumour and liver metastasis by laparoscopic approach has been reported[56] (Table 2).

LLR for hepatocellular carcinoma: When resection for HCC is discussed, an important aspect is the state of the liver, as clearly, incidence of HCC is vastly increased among cirrhotics. However LLR in cirrhotics is discussed elsewhere and emphasis here is placed on analysis of studies comparing LLR to OLR for HCC in terms of survival. Most of them support the use of LLR with no significant differences in 5-year overall survival (61% *vs* 62%) or disease-free survival (31% *vs* 29%) between the LLR and OLR groups[51]. Similarly Nguyen *et al*[3] showed, 5-year overall (OS) and disease-free survival(DFS) rates after LLR for HCC to be 50%-75% and 31%-38.2%, respectively. One situation where LLR might be of advantage is when the possibility of salvage transplant after resection exists as post-operative adhesions are clearly reduced after a laparoscopic procedure when compared to open[57]. In the presence of portal hypertension, adhesiolysis is not just tiresome, but can cause significant haemorrhage and morbidity[58] (Table 3).

LLR in patients with cirrhosis: Given the incidence of HCC in patients with cirrhosis, clearly a large proportion of patients who come for liver resection will be cirrhotic. Buell *et al*[2] had by 2008 performed about 500 hand-assisted LLR, of which 31 were in the presence of cirrhosis. These resections included 18 segmentectomies, 6 bisegmentectomies, 4 left lateral segmentectomies, 2 left lobectomies, and 1 central hepatic resection. No right hemihepatectomies were attempted in cirrhotics[4]. On review of more recent series that deal with HCC only, the percentage of cirrhotic resections would be expected to be higher, and in reality ranges between 45% and 100%[49,59]. Hence the outcome of LLR in these selected patients is important to note. There are several reasons for LLR to be complicated by cirrhosis: (1) the stiff liver is difficult to manipulate; (2) presence of portal hypertension; (3) underlying clinical or sub-clinical coagulopathy which is often not easy to control accurately; (4) deep tumours or lesions might be hard to palpate when compared to normal pliable livers; (5) pneumoperitoneum with its impact on portal flow, might have a unpredictable influence on post-operative liver function; (6) a fibrotic liver is likely to increase overall bleeding as the stiff, deranged architecture does not allow vessels to collapse/constrict when injured as they might in normal tissue; and (7) last but not least, patients with chronic liver disease are less likely to tolerate complications when compared to patients with no liver disease and indeed need a greater future liver remnant. However a review of the literature however shows that LLR in cirrhotics is indeed safe and feasible[12,60-63].

In fact these studies uncovers several important find-

Table 4 Some of the larger laparoscopic donor hepatectomy series wherein operative time, transfusion, blood loss, and hospital stay are discussed

Ref.	Method	*n*	Operative time (min)	Blood Loss (mL)	Transfusion	Conversion	Donor Morbidity	Hospital stay (d)
Soubrane *et al*[69]	Pure laparoscopy LLS	16	320 ± 67 *vs* 244 ± 55	18.7 ± 44.2 *vs* 199.2 ± 185.4	NIL	1/16	18.7% *vs* 35.7% (NS)	7.5 ± 2.3 *vs* 8.1 ± 3.0 (NS)
Kim *et al*[70]	Pure laparoscopy LLS	11	330 ± 68 *vs* 306 ± 29	396 ± 72 *vs* 464 ± 78	NIL	-	0/11 *vs* 1/11	6.9 ± 0.3 *vs* 9.8 ± 0.9
Koffron *et al*[16]	Lap assisted Right hepatectomy	20	-	-	-	-	-	-
Wakabayashi *et al*[83]	Lap assisted Right hepatectomy	7	-	-	-	-	-	
Baker *et al*[78]	Lap assisted Right hepatectomy	33	265 ± 48 *vs* 316 ± 61`	417 ± 217 *vs* 550 ± 305	-	-	21.3% *vs* 21.3%	4.3 *vs* 3.9
Eguchi *et al*[79]	Hybrid Lap Assisted (Both Lobes)	15 (6-RL, 9-LL)	456	NA	NA	-	6.60%	-
Marubashi *et al*[89]	Hybrid Lap Assisted (Left lobe)	31 (LLS-17, LL-14)	510 ± 90	353 ± 396	-	NIL	6.70%	10.3 ± 3.3
Choi *et al*[91]	Lap Assisted and Single Port Lap Assisted	60 20(LADH) 40(SPLADH)	278 ± 72.25(SPLADH) 383.55 ± 41.73(LADH)	450 ± 316.43 (SPLADH) 873 ± 653.01 (LADH)	0.25 ± 0.81 (SPLADH) 1.25 ± 2.05 (LADH)	4/60	12/60	-

ings that suggests that LLR is better than OLR in these patients in term of reduced post-operative ascites, electrolyte disturbances, operating time and blood loss apart from the other more routine benefits of laparoscopy[60]. Clearly, outcomes are also related to severity of liver disease and hence we reviewed LLR series that dealt with Child's B/C patients. Morise *et al*[63] in 2011 showed that even in this unwell cohort of patients (with ICG retention > 40% at 15 min), LLR outcomes were favourable, as did Hosokawa *et al*[64].

Conclusion: Therefore in our opinion, in addition to benign liver disease, LLR is clearly safe in malignancy and achieves equivalent outcomes in terms of resection margins and tumour recurrence compared to OLR with similar benefits as detailed several times above. Incidences of port site or peritoneal recurrences appear insignificant. Despite the technical difficulties posed by cirrhosis for LLR, post-operative outcomes in cirrhotic patients after LLR appear to be better than after OLR, presumably due to less frequent liver decompensation which might be related to less venous collaterals being divided, and reduced inflammatory response after LLR. Hepatotoxic pre-operative chemotherapy did not seem to worsen outcomes in the CRLM cohort. Amongst HCC patients who have undergone resection, fewer adhesions (seen after LLR) in the eventuality of a salvage transplant seems attractive.

MILR IN HEALTHY LIVING DONORS, FOR IMPLANTATION

Open donor hepatectomy(ODH) in live donors substantially affects quality of life, with the patients often developing wound related issues and symptoms such as infection, pain, and deformity[65,66]. A recent report of donor morbidities in Japan showed that the incidence of donor surgery-related morbidities was 8.4% in total, and the leading morbidity was bile leak (2.6%), followed by wound infection (1.2%)[67]. As discussed in the sections above, over 150 publications have shown the safety and efficacy of LLR and collectively nearly 3000 LLR have been performed for benign and malignant tumors with a reported peri-operative mortality of 0.3% and morbidity of 10.5%[2,3]. This combined expertise has led surgeons to consider applying these techniques to a living donor setting with the hope of improving the 'donor experience' and the first laparoscopic left lateral donor hepatectomy(DH) was reported by Cherqui *et al*[68] in 2002, throwing open the most controversial topic in LLR. Given the limited worldwide experience in minimal assess (MA) donor hepatectomies (MADH) to date, we reviewed both hybrid/hand assisted laparoscopic DH (HALDH) techniques as well as the pure laparoscopic approach (LDH) (Table 4).

Laparoscopic left lateral segmentectomy

Up until recently all the pure LDH were Laparoscopic left lateral segmentectomy (LapLLS), mainly by two groups, the French (Cherqui D, Sourbane O, *etc*.) and Korean (Lee SG, Kim KH, *etc*.) who between them performed about 25 LDH. With no mortality in either series, comparable morbidity and quicker recovery times, they separately concluded that this is a feasible and reproducible procedure in selected centres[68-71]. First described in 1996, left lateral sectionectomy (LLS) is one of the most anatomic resections in liver surgery and hence benefits from the most standardized laparoscopic surgical approach[1,37].Understandably it is clearly associated with a lower rate of complications and mortality than other donor hepatectomies[16,27,33,69,72-76]. The results are on par with the open procedure with acceptable outcomes. Several studies have shown its advantage over traditional open surgery in reducing the physical and emotional stress experienced by patients[70,77,78].

Surgical technique for left lateral sectionectomy for donation: Usually performed with the patient in supine and 30° anti-Trendelenburg position, and the surgeon standing between the patient's legs (French position), utilising 4 ports [one 5 mm-irrigation and aspiration; one 10 mm-harmonic scalpel (Ultracision; Ethicon Endosurgery, Cincinnati OH, United States) or surgical aspirator CUSA Excel (Integra Life Science Ltd., IDA Business and Technology Park, Ireland); and two 12 mm - 30° optical device, linear stapler). Carbon dioxide pneumoperitoneum is kept at about 10 mmHg to reduce the risk of CO_2 gas embolism.

The left lobe of liver is mobilised, hilar dissection done and the left hepatic artery and the left portal vein dissected and looped. The portal venous branches to the caudate lobe are dissected, clipped and divided. The liver parenchyma is divided on the right side of the round and falciform ligaments with a harmonic scalpel and an Ultrasonic dissector without vascular clamping. As in open resections, the central venous pressure (CVP) is kept as low as possible, to decrease blood loss from the divided parenchyma. Unfortunately, due to 10-12 mmHg pressure pneumoperitoneum, CVP readings are not reliable during LLR and hence readings prior to insufflation and large visual fluctuations of the venacava with respiration movements serve as useful surrogate estimates of filling pressures. Bleeding during transection is controlled with bipolar electrocoagulation and clips. We divided pedicles to segment 4 during transection using a linear stapler (EndoGIA, Tyco, United States). When the dissection reached the hilar plate, the left biliary duct is cut with a curved sharp scissors in a straight line transversely, and the proximal end is closed with a running suture (PDS 5/0). Once the transaction is complete, a Gelport laparoscopic system (Applied Medical, Rancho Santa Margarita, CA, United States) is inserted to allow hand extraction of the graft through a Pfannestiel incision. Then before inflow occlusion 5000 U of heparin is given intravenously. With double Hem-o-lock clips (Weck Surgicals, Teleflex, United States) on both the hepatic arteries (Endo TA, 30 mm; Autosuture, United States) and on the left portal vein, the EndoGIA (45 mm) was used to secure and cut the left hepatic vein. The graft is retrieved through the Pfannestiel incision and perfused with cold University of Wisconsin Solution in the standard manner. Once retrieved, the Pfannenstiel incision is closed, pneumoperitoneum re-established and hemostasis secured. The cut surface is visualised for any bile leaks and if found, suture ligated before removing ports under vision. Many laparoscopic centres including ours do not place abdominal drains after left lateral resection.

Hand/hybrid assisted laparoscopic donor hepatectomy surgery: In addition to the LDH experience, between 2006 and 2013 there have been several reports on hand/hybrid assisted laparoscopic donor hepatectomy surgery (HALDH)[16,71,78-83]. Hand assisted or Hybrid technique refers to the placement of an additional hand port through a 10-12 cm incision, through which the surgeon manipulates the liver for mobilization, parenchymal transection and control of bleeding. The devices commonly used are a Gelport (Applied Medical, Rancho Santa Margarita, CA) which is placed through a 6-8 cm incision. The incision can be sited at different locations based on the lobe being harvested (26-29). Right lobe HALDH is advocated by some groups in order to reduce donor morbidity and faster recovery.

Of the authors of 3 of the larger series Baker, Wakabyashi and Koffron have performed collectively, approximately, 60 right hepatectomies *via* HALDH. None have reported mortality, and overall morbidity was comparable to the open approach, with the added advantage of faster recovery and reduced blood loss[16,78,83]. Although the purists feel hand ports remove the advantage of MILR surgery, these reports confirm better recovery, shorter stay even in this group of patients when compared to the open cohort[71,80-82]. Moreover on analysing papers that combine data on both pure LDH and HALDH, there appears to be an advantage in utilising these procedure sequentially, to safely ease one through the laparoscopic learning curve, working towards a pure laparoscopic approach[16]. On a contentious note, some authors feel that given the need for the graft to be removed without damage, pure LDH for a large right lobe can only have a limited benefit. According to Ha *et al*[71], a 10-cm-long incision is needed to deliver a small-sized right liver graft of 500 g, and a 700 g graft requires an incision of up to 12 cm. Owing to this inevitable limit of the skin incision, they go as far as to say that a totally laparoscopic approach is not suitable when harvesting right liver grafts in contrast to small grafts which can be delivered through a transverse supra-pubic incision.

Nevertheless, we note in the current issue of the *American Journal of Transplantation*, three articles that describe pure LDH approach to left and right lobe grafts[84-86]. These 3 groups separately, successfully com-

pleted 7 pure LDH (1 right DH) between them with one graft failure, due to an intra-hepatic abscess. Clearly the expertise required was carefully gained over several years as all of these centres have considerable experience in both LLR and liver transplantation. In our opinion clearly these landmark papers answer the first "Can we do it?" question. However when assessing MADH, the second question of "should we do it?" is more difficult to answer and two concerns take precedence, one graft outcomes (considering the "specimen" is expected to function normally after extraction and implantation) and perhaps more importantly, donor safety (considering that DH is peculiar in that healthy humans are exposed to the risk of death).

Graft outcomes: Based on a report by Thenappan *et al*[87] in 2011, early graft function is at least as good if not better in grafts procured through the MADH approach. Thenappan *et al*[87] compared 15 MA to 15 open donor hepatectomies with regards to peak bilirubin, AST and INR levels, up to 90 d after transplant and found no significant differences except a minor increase in INR on day 7 in the MA group versus the open group. In fact a trend towards a decrease in biliary and vascular complications was noted (NS), with a one year graft and patient survival of 100% in the MADH group. One could speculate that the closer, magnified vision possible with the laparoscope that enabled better intra-operative detection of bile leaks accounted for the decrease in biliary complication rate. The inevitable increase in warm ischemia time (WIT) was thought to be a potential issue with MADH. However Soubrane *et al*[69] counter-intuitively showed no significant difference in WIT, but more significantly, no appreciable difference in graft function with the laparoscopic approach. However in this 2006 French paper, of the 16 recipients(All LLS) in the LLR group, there were 2 hepatic artery thrombosis (HAT) and one portal vein thrombosis compared to one each among the 14 open donor hepatectomies (overall rate of HAT in group-3 /30 patients, which is higher than most published reports). With small numbers and short follow-up it is difficult to draw conclusions from this data, and neither have the authors volunteered a possible explanation for this high thrombosis rate.

Donor safety: The second concern of donor safety is more difficult to address given the limited experience. A recent worldwide survey of ODH revealed that the donor mortality rate was 0.20% (23/11553), with 19 of these 23 deaths related to the actual procedure[88]. In addition, potentially life-threatening near-miss events were reported, with an incidence of not less than 1.1%. Clearly whilst considering MADH, we must not underestimate such "near-miss" events that are unlikely during open hepatectomy, *e.g.*, trocar injury. Of the several original articles on MADH to date, none have reported mortality, and overall morbidity was said to be comparable to the open approach, with the added advantage of faster recovery and reduced blood loss[16,69,70,78,83]. However in the Marubashi *et al*[89] study evaluating the efficacy of HALDH in 31 donors (17 left lateral donation and 14left lobes), short term outcomes were compared with historical open hepatectomy groups when despite lack of mortality, there were two major morbidities in laparoscopic group, one a diaphragmatic injury and the other, an injury to the right hepatic vein requiring conversion for control. Nonetheless, all donors made early recovery, and assessment by SF36-v2 questionnaire revealed early recovery of all components. As seen with the advent of donor nephrectomy, given the results of more experienced centres, despite occasional mishaps, one might expect numbers to build as time passes, leading to such procedures becoming standard operations. But despite the results discussed above, concerns about technical management of sudden blood loss during liver transaction, length of graft vessels available for implantation (especially extra-hepatic length of the hepatic vein) appear potential issues to the uninitiated. It is reassuring that none of these reports have detected post-operative venous outflow issues during follow-up.

Special issues in MADH-donor selection, liver regeneration: Given the peculiarities of LDH, it makes sense to have at least loose criteria to try and select the perfect candidates for the MADH approach apart from the type of resection. In our opinion, in addition to the safety criteria that is currently in use for the open approach (age between 18-45 years, medically fit *etc.*), graft steatosis less than 10% (to allow better toleration of portal flow disturbances), estimated graft weight less than 700 g, and conventional hepatic, portal and biliary anatomy, appear at least for the time being reasonable criteria. Avoidance of donors with IRHV and abnormal anatomy allows standardisation of the procedure and reduces operating time. In terms of donor body mass index (BMI) most programmes have a upper limit of 27 or 30 to qualify for donation surgery. However in contrast to the open approach, in those unusual donors in whom despite a high BMI (> 30), liver steatosis appears < 10%, the LDH route appears particularly suited. Interestingly, liver regeneration is a crucial aspect in returning living donors to their pre-morbid state. Although this phenomenon has not been completely studied or understood, there is some evidence that regeneration after MADH is better when compared to the open technique [86% *vs* 73% regeneration at 3 mo (P = 0.03)][78]. Many prior studies have established a diminished acute-phase stress response and improved immune system function after laparoscopic surgery including 1 study in an animal model of liver resection[90]. Clearly this is a significant potential advantage that needs further study in a randomized manner.

CONCLUSION

In conclusion LDH is still a controversial indication for LLR and must be "*self-restricted*" to centres performing at

least 2 such procedures a month. Laparoscopic left lateral sectionectomy has been well validated and has been reproduced by many centres. Laparoscopic assisted or pure left lobe hepatectomy is attractive too in order to reduce the wound related morbidity and enable faster recovery. Pure laparoscopy for right lobe donation needs to be more carefully evaluated in larger series with post-operative outcome data showing its safety and clear benefits before it can be recommended (especially as the incision required to safely deliver a large graft, might negate the benefits).

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TOPIC HIGHLIGHT

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Diagnosis and management of choledocholithiasis in the golden age of imaging, endoscopy and laparoscopy

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Abstract

Biliary lithiasis is an endemic condition in both Western and Eastern countries, in some studies affecting 20% of the general population. In up to 20% of cases, gallbladder stones are associated with common bile duct stones (CBDS), which are asymptomatic in up to one half of cases. Despite the wide variety of examinations and techniques available nowadays, two main open issues remain without a clear answer: how to cost-effectively diagnose CBDS and, when they are finally found, how to deal with them. CBDS diagnosis and management has radically changed over the last 30 years, following the dramatic diffusion of imaging, including endoscopic ultrasound (EUS) and magnetic resonance cholangiography (MRC), endoscopy and laparoscopy. Since accuracy, invasiveness, potential therapeutic use and cost-effectiveness of imaging techniques used to identify CBDS increase together in a parallel way, the concept of "risk of carrying CBDS" has become pivotal to identifying the most appropriate management of a specific patient in order to avoid the risk of "under-studying" by poor diagnostic work up or "over-studying" by excessively invasive examinations. The risk of carrying CBDS is deduced by symptoms, liver/pancreas serology and ultrasound. "Low risk" patients do not require further examination before laparoscopic cholecystectomy. Two main "philosophical approaches" face each other for patients with an "intermediate to high risk" of carrying CBDS: on one hand, the "laparoscopy-first" approach, which mainly relies on intraoperative cholangiography for diagnosis and laparoscopic common bile duct exploration for treatment, and, on the other hand, the "endoscopy-first" attitude, variously referring to MRC, EUS and/or endoscopic retrograde cholangiography for diagnosis and endoscopic sphincterotomy for management. Concerning CBDS diagnosis, intraoperative cholangiography, EUS and MRC are reported to have similar results. Regarding management, the recent literature seems to show better short and long term outcome of surgery in terms of retained stones and need for further procedures. Nevertheless, open surgery is invasive, whereas the laparoscopic common bile duct clearance is time consuming, technically demanding and involves dedicated instruments. Thus, although no consensus has been achieved and CBDS management seems more conditioned by the availability of instrumentation, personnel and skills than cost-effectiveness, endoscopic treatment is largely preferred worldwide.

Key words: Biliary lithiasis; Choledocholithiasis; Laparoscopy; Endoscopy; Diagnosis; Management

Core tip: Common bile duct stones (CBDS) are not infrequent in patients with gallstones and should be treated. The concept of "risk of carrying CBDS", based

on symptoms, liver serology and ultrasound, is pivotal to identify the appropriate management. While "low risk" patients do not require further examination, "intermediate to high risk" patients may be offered intraoperative cholangiography (IOC) and laparoscopic choledochus exploration, or may be referred to magnetic resonance cholangiography (MRC), endoscopic ultrasound (EUS), endoscopic retrograde cholangiopancreatography and sphincterotomy. Whereas the results of IOC, MRC and EUS are similar in identifying CBDS, surgery seems superior to endoscopic sphincterotomy in choledochus clearance, although this latter is preferred worldwide.

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INTRODUCTION

The prevalence of gallbladder lithiasis is roughly 20.5 million (6.3 million men and 14.2 million women) in the United States[1], whereas in Europe it is reported to vary between 5.9% and 21.9% of the general population[2]. Eleven to 21% of patients with cholelithiasis also have concomitant common bile duct stones (CBDS) at the time of surgery[3-5].

The vast majority of CBDS form within the gallbladder and then migrate into the common bile duct (CBD), following gallbladder contractions. Once in the CBD, stones may reach the duodenum following the bile flow; otherwise, also owing to the smaller diameter of the distal CBD at the Vater papilla, they may remain in the choledochus. In this latter case, gallstones may be fluctuant, thus remaining mostly asymptomatic, or cause a variety of bile flow problems, including complete obstruction and jaundice. Bilostasis may be responsible for bile infection and consequent ascending cholangitis, whereas bile/ pancreatic juice flow problems at the merging of the CBD and the main pancreatic duct (Wirsung) are presumed to potentially trigger the intrapancreatic activation of pancreatic enzymes, thus causing acute biliary pancreatitis[6]. Thus, the clinical presentation of choledocholithiasis may vary widely, as CBDS may be asymptomatic (up to half of cases[7]), or associated with various symptoms and conditions, ranging from colicky pain to potentially life-threatening complications, such as ascending cholangitis or acute pancreatitis (see below).

CBDS diagnosis and management have radically changed over the last 30 years. Since the early eighties, the dramatic diffusion of endoscopic techniques, namely endoscopic retrograde cholangiography (ERC), has changed the approach to patients affected by cholelithiasis, thus potentially carrying CBDS. Until then, a first evaluation was made by clinical symptoms, preoperative ultrasound (US), liver laboratory examinations and systematic preoperative intravenous cholangiography[8], which had replaced oral cholecystography[9]; later, intravenous cholangiograpy became a pre-ERC examination aimed at defining CBDS risk[10,11], and is nowadays replaced by magnetic resonance cholangiography (MRC), endoscopic ultrasound (EUS) and ERC[12-14].

During the 1990s, the diffusion of laparoscopy changed biliary lithiasis management even more radically, by introducing laparoscopic cholecystectomy[15-17], intraoperative cholangiography[18,19] and, when needed, laparoscopic CBD exploration[20-22]. Concomitantly, other techniques were proposed for CBDS management, including lithotripsy[23-25].

During the 2000s, a critical appraisal of management options[26-28] and the diffusion of new diagnostic examinations led to a more cautious, patient-tailored preoperative workup, based on patient risk of carrying CBDS[3,29,30], and management, based on the perception that CBDS may be treated in a multidisciplinary way[31-33]. Moreover, the categorical diktat to treat any patient with CBDS stones (even asymptomatic), which was still incorporated in international guidelines at least until the late nineties[34], is nowadays challenged by a more conservative attitude, according to the belief that in over one third of cases CBDS will finally pass through without complications[31,35,36]. Interestingly, the 2006 European Society for Endoscopic Surgery (EAES) guideline update justified an expectant attitude in elderly patients[37], whereas, in 2008, the British Society of Gastroenterology guidelines[38] recommended that whenever patients have symptoms and investigations suggest ductal stones, extraction should be performed when possible (on the strength of supporting evidence grade Ⅲ; recommendation grade B). In 2011, the American Society for Gastrointestinal Endoscopy (ASGE) guidelines[39] incorporated those recommendations with a "low quality of evidence".

Such considerations gain importance for asymptomatic CBDS, which represent up to roughly one half of cases[7] and where aggressive procedures may appear unjustified[40]. Asymptomatic CBDS are actually the most challenging situation, since patients risk being "understudied" by poor diagnostic work up or "over-studied" by excessively invasive examinations. Similarly, they can be under- or over-treated by inappropriate procedures. Two main open issues remain without a clear answer: how to cost-effectively diagnose CBDS and, eventually, how to deal with them. In this light, the concept of "risk of carrying CBDS" is pivotal to identifying the most appropriate algorithm of management of a specific patient[29,41-44].

Two main "philosophical approaches" face each other nowadays, at least for patients with an intermediate to high risk of carrying CBDS: on one hand, the "laparoscopy-first" approach, which mainly relies on intraoperative cholangiography and laparoscopic CBD exploration, and, on the other hand, the "endoscopy-first" attitude, variously referring to MRC or EUS for diagnosis and ERC fol-

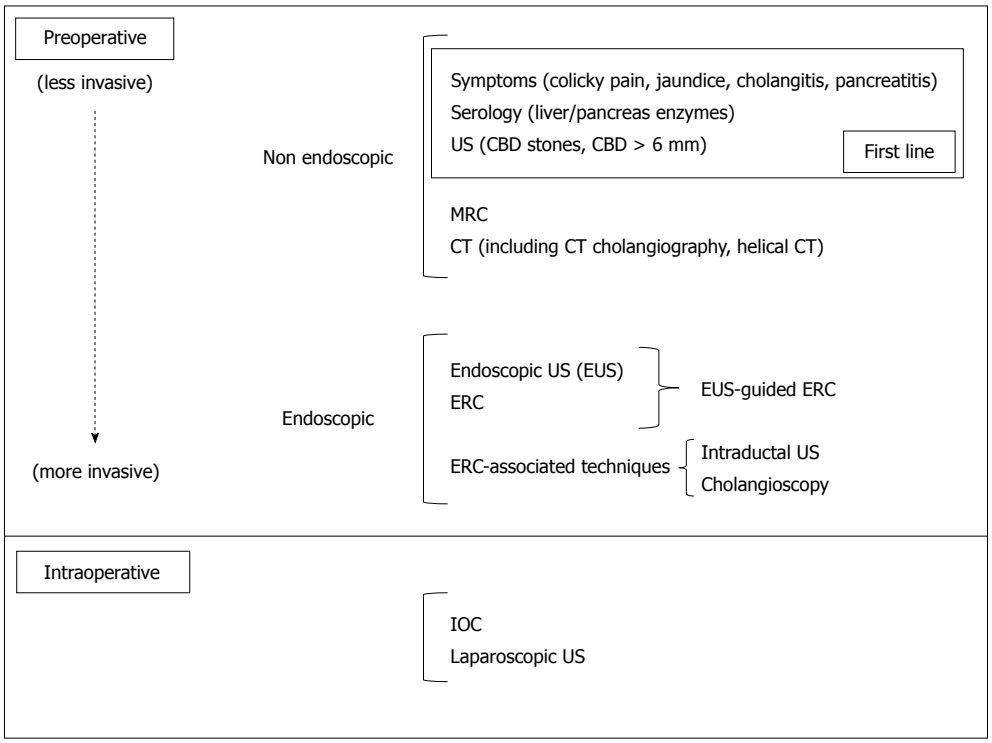

Figure 1 Clinical symptoms, serology and imaging options for the diagnosis of common bile duct stones. US: Ultrasounds; MRC: Magnetic resonance cholangiography; CT: Computerized tomography; ERC: Endoscopic retrograde cholangiography; IOC: Intraoperative cholangiography; CBD: Common bile duct.

lowed by endoscopic sphincterotomy for CBD clearance. As no consensus has been achieved, CBDS management seems more conditioned by availability of instrumentation, personnel and skills than cost-effectiveness.

Here we propose a review of the most widely diffused approaches to CBDS diagnosis (Figure 1) and management (Figure 2).

DIAGNOSIS

Diagnostic tools are reported as summarized in Figure 1 in a chronological order (1) Preoperative; and (2) Intraoperative, from the least to the most invasive.

In order to understand why there is still no consensus concerning CBDS diagnosis and so many examinations have been and are continuing to be proposed, the following statements should be considered: The prevalence of gallbladder stones in the general population is up to 20%[2]; of these patients, up to 20% have synchronous CBDS[5]; CBDS are asymptomatic in up to half of these latter cases[7]. These data mean that up to 2% of the general population may have unknown CBDS during their life-span; CBDS may cause potentially life-threatening complications, such as acute cholangitis or acute pancreatitis, and therefore should be diagnosed and treated[29,34,37-39]; Accuracy, invasiveness, potential therapeutic use and costs of the most common imaging techniques used to identify CBDS increase together in a parallel way: they are minimal for transabdominal US and maximum for ERC, where the counterpart of intrinsic therapeutic implications (endoscopic sphincterotomy) are non-negligible morbidity/mortality[14,26]; These latter considerations contraindicate the systematic, pre-cholecystectomy use of imaging techniques other than transabdominal US as first-line imaging, and ERC as second-line examination, unless a clear indication is given by jaundice, cholangitis or high risk of synchronous CBDS[29,38] (Table 1).

Here we propose a review of the most widely diffused techniques used to ascertain the presence of CBDS.

PREOPERATIVE EXAMINATIONS

Non-endoscopic examinations

Symptoms: Characteristically, the symptom complex of choledocholithiasis consists of right upper abdominal colicky pain, radiating to the right shoulder with intermittent jaundice accompanied by pale stools and dark urine[45], whereas cutaneous itching is rarely present[46]. In contrast with patients with neoplastic obstruction of the CBD or ampulla of Vater, the gallbladder is usually not distended (Courvoisier's law)[45]. Scholastically, Charcot's triad[47] (jaundice associated to biliary colic pain and sepsis - hyperpyrexia and chills), indicates acute cholangitis; as choledocholithiasis is the most frequent aetiology of such a clinical picture, it should prompt immediate diagnostic confirmation and CBD drainage[38]. Acute pancreatitis, showing with transversal abdominal pain potentially irradiated to the back and associated to an increase of serum level of amylase/lipase, in the presence of gallstones, is a priori considered as being of biliary origin. Hepatic abscess(es) may also be a rarer infectious complication of CBDS whereas chronic CBD obstruction may also cause biliary cirrhosis[38]. All the reported clinical pictures should lead us to focus on the right upper quadrant in

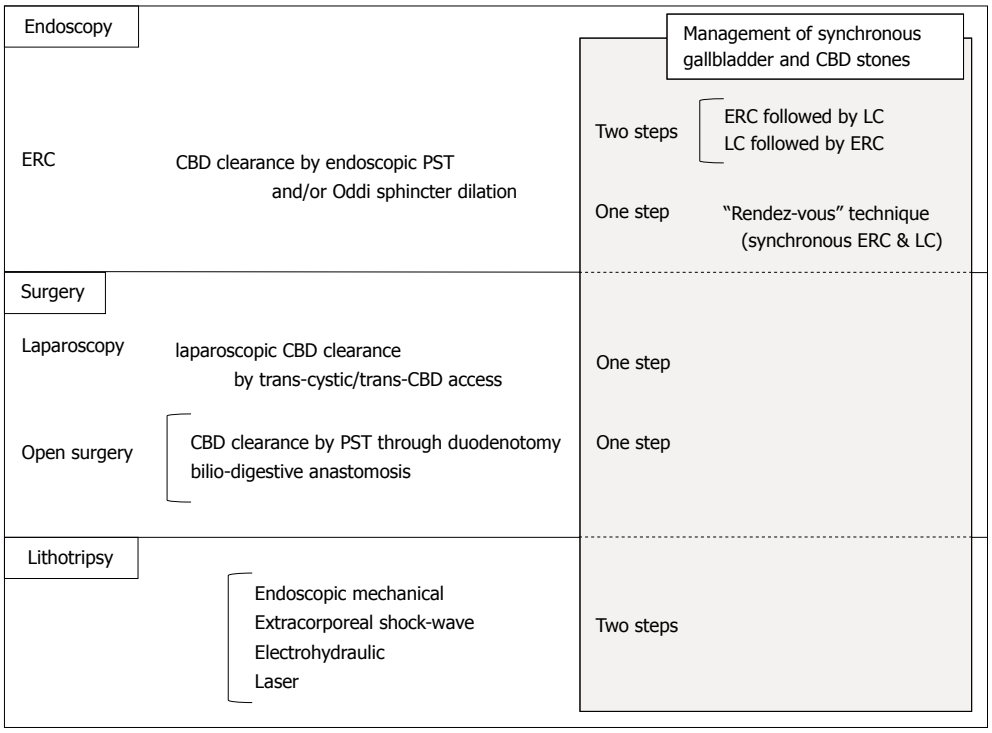

Figure 2 Management options for common bile duct stones. CBD: Common bile duct; ERC: Endoscopic retrograde cholangiography; LC: Laparoscopic cholecystectomy; PST: Papillosphincterotomy.

Table 1 Common bile duct stones diagnosis and management: Current evidence

Biliary lithiasis affects 10% to 20% of general population and is associated with CBDS in up to 20% of cases
Clinical symptoms, liver/pancreas serology and transabdominal ultrasounds may define the "risk of carrying CBDS", and identify:
"Low risk" patients, to be directly referred to laparoscopic cholecystectomy
"Intermediate risk" patients, needing intraoperative cholangiography, endoscopic ultrasounds or magnetic resonance cholangiography before laparoscopic cholecystectomy
"High risk" patients requiring endoscopic retrograde cholangiography
CBDS may be managed by endoscopic sphincterotomy or surgery (laparoscopic or open). This latter has seemingly slightly better results, counterbalanced by invasiveness (open surgery) or the need of specific instrumentation and advanced laparoscopic skills (laparoscopic surgery). Lithotripsy may help endoscopic CBDS retrieval or may be performed extra-corporeally in selected, unfit patients
CBDS management will be more and more multidisciplinary and tailored not only on a specific patient but also on the available resources of a specific environment to have the best possible management

CBDS: Common bile duct stones.

order to identify biliary lithiasis as possible aetiology.

Serology: Traditionally, an alteration of the so-called cholestasis indexes (direct bilirubin, gamma-glutamil-transpherase, alkaline phosphatase)[44,48] was considered as potentially due to CBDS. Indeed, also liver cytolysis indexes were also found to be associated to unknown CBDS[3,44,49]. Nowadays, although total bilirubin is considered the main laboratory index related to the risk of synchronous CBDS ("very strong predictor")[29] and its ensuing management, all liver biochemical tests other than bilirubin deserve a careful evaluation ("moderate predictors")[29].

Transabdominal US: Transabdominal US represents the first line, non-expensive, non-invasive imaging examination available in virtually any environment[29,50,51], performed for any sign/symptom/condition possibly referring to liver disease. The small distance of the gallbladder from the abdominal wall and the absence of interposed gas make transabdominal US the ideal examination to study gallbladder morphology and to ascertain the presence of gallstones, where sensitivity of US is 96%[52].

Unfortunately, US accuracy in detecting CBDS drops to less than 50%[52,53], since CBDS often do not show acoustic shadowing or are located in the distal part of the CBD, where they are often obscured by gas[53]. In those cases, CBDS diagnosis often relies on indirect signs of CBD obstruction, such as CBD dilation. The definition of CBD dilation is also a matter of discussion, as suggested "normal limits" vary widely, ranging from 5 to 11 mm[7,54,55], partly because CBD diameter may increase with age and after cholecystectomy. The accuracy of US in visualizing the gallbladder and its content may allow

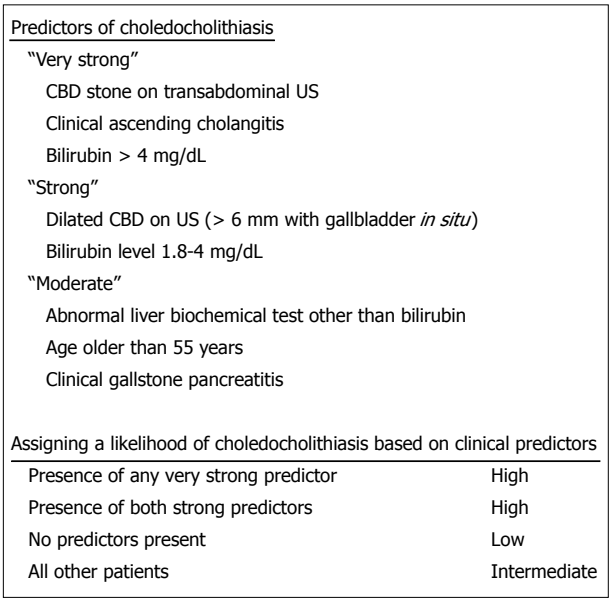

Figure 3 The American Society for Gastrointestinal Endoscopy estimation of risk of carrying common bile duct stones in patients with symptomatic cholelithiasis based on clinical predictors[39]. US: Ultrasounds; CBD: Common bile duct. With the permission of the journal *Gastrointestinal Endoscopy*.

us to identify another indirect sign of increased CBDS risk: the number and size of gallbladder stones[51,56]. As multiple, small-sized gallstones are more likely to migrate into the CBD[43,57], this US finding should be considered during CBD assessment.

Scoring systems and algorithms: The above mentioned "first-line" diagnostic criteria have been variously associated to identify patients at high risk of carrying CB DS[3,43,44,48,49,58,59]. Although they mostly showed the statistical significance of clinical, laboratory and US findings, nevertheless, the extreme variability of proposed models and the doubtful results of some studies[60] have limited the use of scoring systems and algorithms.

Although none of those scoring systems have ever really entered clinical practice on a large scale, they[41,42,48] have somewhat paved the way to recent guidelines[29], which have identified three classes of risk of CBDS based on symptoms, liver serology and transabdominal US. The risk of carrying CBDS is defined as low where no other examination is needed, intermediate where preoperative EUS/MRC or laparoscopic intraoperative colangiography (IOC)/US is requested, and high where patients should be directly referred to preoperative ERC (and possibly ES). The proposed algorithm based on the predictors listed in Figure 3 and is reported in Figure 4.

CT scan: CT scan is a "second line examination" for many abdominal diseases/conditions, partly owing to patients' exposure to X-rays and higher costs compared to US. Traditionally considered more accurate than trasparietal US in identifying CBDS[61] but still inferior to MRC[62], recent advances, such as the "helical" CT scan[29,63] and CT cholangiography[46,62], have increased its accuracy. CT-cholangiography, performed after the administration of iodinated contrast agent excreted into the bile, has shown a sensitivity (88%-92% *vs* 88%-96%) and specificity (75%-92% *vs* 75%-100%) comparable to MRC[46,62]. New generation 64-detector CT at the portal venous phase is reported to have a 72%-78% sensitivity and a 96% specificity in identifying CBDS[63]. Very recently, 64-sliced CT was reported not to detect CBDS in 17% of cases, stone size < 5 mm, color black/brown and patient's age being related to non-detectability at multivariate analysis[64]. Although the intrinsic higher "invasiveness" of CT compared to MRC makes the latter preferable nowadays, the extreme availability and diffusion of CT scan may possibly lead to its rediscovery in this field.

MRC: MRC is considered nowadays to be the most accurate non-invasive (non-endoscopic) procedure for the detection of CBDS, with 85%-92% sensitivity and 93%-97% specificity in large series[12,65,66]. A recent meta-analysis showed that the aggregated sensitivities of EUS and MRC were 93% and 85% respectively, whereas their specificities were 96% and 93% respectively, with no significant differences[12]. Regardless of overall effectiveness, it should be pointed out that MRC is a non-invasive technique, which may allow for providing a higher spatial resolution than EUS, but is probably less sensitive than EUS for detecting CBD stones smaller than 6 mm[66]. Other drawbacks of MRC are its suboptimal availability in non-tertiary care centers, and non-therapeutic purpose of the technique, which involves a further procedure to eventually treat CBDS. The impossibility of its use in specific situations (morbid obesity or claustrophobic patients, the presence of metal foreign body/device) is also a specific issue of this technique.

Endoscopic examinations

All endoscopic procedures share the pros and cons of peroral endoscopy. Although endoscopically reaching the second duodenum is widely considered to be an easy task for average endoscopists in average patients, some conditions can make this manoeuvre a difficult one. In some cases, the papilla major is difficult to identify and to cannulate, resulting in a challenging situation for the endoscopist (and dangerous for the patient), as it is for example when it is placed in a duodenal diverticulum[67]. Previous surgical procedures on the stomach, such as Roux-en-Y gastric by-pass[68] or gastrectomy with duodenal stump closure and Billroth II reconstruction[69], are another frequent cause of ERC failure and complications[70]. In particular, endoscopically reaching the second duodenum is difficult after a Roux-en-Y reconstruction, but also after an "Ω"-shaped anastomosis. In those cases, both CBDS diagnosis and management have to be carried out surgically (open surgery or laparoscopy).

ERC: After having been widely used for CBDS diagnosis in the late eighties/nineties, with a 75%-93% sensitiv-

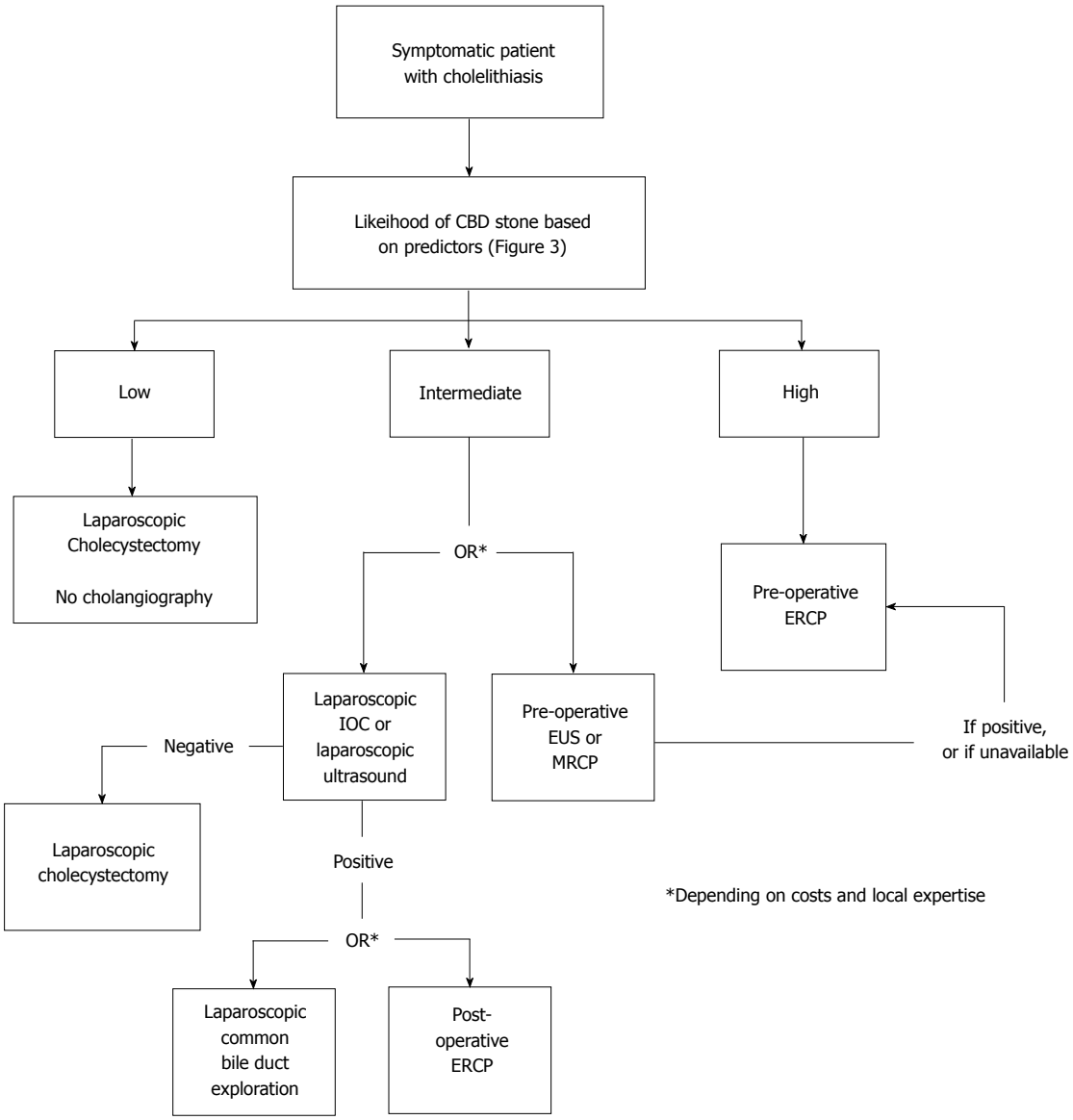

Figure 4 The American Society for Gastrointestinal Endoscopy algorithm for the management of patients with symptomatic cholelithiasis based on the degree of probability for choledocholithiasis[39]. CBD: Common bile duct; IOC: Intraoperative cholangiography; EUS: Endoscopic ultrasounds; MRCP: Magnetic resonance cholangiopancreatographygraphy; ERCP: Endoscopic retrograde cholangiopancreatography. With the permission of the journal *Gastrointestinal Endoscopy*.

ity and 100% specificity[14,71,72], ERC is nowadays being progressively abandoned as a diagnostic tool for patients with moderate to intermediate risk of carrying CBDS, as most cholangiograms result as being normal[73] and ERC is not cost-effective[74]. Compared to EUS and MRC, accuracy of ERC is suboptimal, being reduced in the case of CBD dilation and small CBDS[14]. Moreover, ERC not only involves X-ray exposure and the intrinsic invasiveness of endoscopy, but it also has non-negligible procedure-related morbidity/mortality[26,75-77], with a 2%-11% acute pancreatitis rate[13,14,78]. Moreover, once ERC is performed, endoscopic sphincterotomy is frequently associated regardless of CBDS presence, both for the risk of a post-ERC ascending cholangitis and for the possible false negatives of ERC itself. Such a practice of performing endoscopic sphincterotomy systematically after ERC is obviously a good argument against a supposed lesser invasiveness of diagnostic ERC compared with "operative" ERC. Thus, while ERC remains indicated for patients with a high risk of presenting synchronous CBDS, MRC, EUS or IOC are nowadays preferred for intermediate risk cases[29].

EUS and EUS-guided ERC: EUS is increasingly performed worldwide as a diagnostic tool, often as the first step of a potential double-technique procedure (EUS and ERC/ES)[13,14]. On the basis of the scientific evidence of no statistically significant difference in sensitivity (93% *vs* 85%) and specificity (96% *vs* 93%) between EUS and MRC[12], ASGE guidelines[29] have proposed MRC or EUS in patients at intermediate risk of CBDS. Nevertheless, the two techniques have particular pros and

cons. Although EUS optimizes the low impact of US associated with the potential therapeutic option of ERC (namely ES), it involves endoscopy under sedation/anesthesia, thus being intrinsically more invasive than MRC. Compared to ERC, EUS has shown the same specificity and higher sensitivity (91% *vs* 75%), mostly due to cases with CBDS smaller than 4 mm or dilated CBD, where ERC presented some false positives[14]. Moreover, EUS has very limited (2%-8%), low degree morbidity, with a virtually 0 post-procedure acute pancreatitis[13,14,79]; thus, it may enable us to avoid ERC-related morbidity/mortality whenever unnecessary, as it is in 2/3 of cases[80], without increasing the risk of further endoscopic procedures in patients with an intermediate risk of carrying CBDS[13,14]. The (moderately) invasive nature of EUS, and the need for an endoscopic theatre, including instrumentation, personnel and expertise, make this option optimal whenever the risk of having CBDS is high enough to allow patients to potentially take advantage of contextual EUS/ERC/ES. In the latter case, EUS and ERC done in a single session proved to be safe, with no increase in sedation- or procedure-related complications, nor need for further endoscopic procedures[14,81], whereas postponing treatment for symptomatic CBD stones exposes the patient to biliary complications, especially cholangitis[12]. Ideally, future guidelines should take into account the specific pros and cons of MRC and EUS, thus defining subgroups of patients as being candidates for one procedure or the other.

ERC-associated intra-, extra-ductal US and cholangioscopy: During the 2000s, high-frequency (12.30 MHz) intraductal US was proposed for a further increase of ERC accuracy[72,78]. As the procedure is wire-guided, the cannulation rate of papilla major is reported to be virtually 100%[72] and sensitivity 97%-100%[72,78]. In particular, intraductal US is reported to have a 100% sensitivity in identifying non-radio-opaque stones, which may pass undiagnosed by traditional imaging[78]. Intraductal US is also reported to identify 100% of CBDS undetected at ERC[82] and 24%-40% of post-ERC/ES residual stones[82,83], thus allowing for definitive CBD clearance, by saline solution irrigation[83], and reduced CBDS recurrence[83].

Recently, extraductal endoscopic ultrasonography has been reported to have the same sensitivity as traditional EUS (90% *vs* 92%); since it can be performed with a traditional duodenoscope using miniprobes, it presents the advantage that, if therapeutic intervention becomes necessary, there is no need to change the scope[84].

Endoscopic cholangioscopy has also been reported to potentially increase the accuracy of ERC by detecting undiagnosed residual CBDS in 23% of ERC-negative cases[85]. State-of-the-art, ultra-slim choledochoscopes allow for magnification of intraductal imaging, differential diagnosis and stone management by basket/balloon retrieval or lithotripsy[85,86]. Although promising, these latter technologies still have a limited role in CBDS management, partly because of the need for costly instrumentation and adequate training of teams, and the uncertain clinical relevance of undiagnosed residual CBDS after ES.

INTRAOPERATIVE EXAMINATIONS

All the intraoperative techniques aimed at identifying CBDS share advantages and drawbacks: on one hand, they avoid any preoperative procedure, thus potentially reducing hospitalizations, hospital stay and costs; on the other hand, they inevitably reduce the therapeutic options whenever CBDS are finally identified at surgery, since any preoperative procedure is obviously not possible anymore. The remaining options include laparoscopic CBD clearance[22,75], synchronous ERC[87,88] or postoperative ERC[31,75]. The first two options involve specific, dedicated instrumentation and surgical/endoscopic skills, and the third one has the main drawback that, whenever ERC fails (previous gastrectomy,..), a third procedure is finally needed to treat CBDS[32]. It should be noted that this latter procedure, in most environments, is an "old-fashioned" CBD exploration by laparotomy (and duodenotomy or biliodigestive anastomosis), which de facto cancels any effort to treat CBDS mini-invasively. Similarly, intraoperative techniques present the common issue of what to do whenever they are not possible or not conclusive, putting the surgeon in a challenging situation to decide whether to explore the CBD (laparoscopically or by open approach).

IOC

First reported in general surgery in the 1930s[89] and introduced in the laparoscopy armamentarium in the early nineties[18], IOC is still under debate regarding its cost-effectiveness when performed systematically or in a selected population[27,30]. IOC substantially relies on low cost laparoscopic instruments and the availability of a mobile roentgenography, and does not require advanced laparoscopic skills. Considering that it is reported to have a 59%-100% sensitivity and a 93%-100% specificity for CBDS[19], its cost-effectiveness is a strong argument in favour of its systematic use. In contrast, a recent systematic review of IOC did not show any benefit of performing IOC routinely[90], somewhat supporting ASGE Guidelines[29] indicating IOC for patients at intermediate risk of carrying CBDS, to eventually treat laparoscopically[22,27] or to refer for postoperative ERC[30]. Moreover, IOC involves the cannulation of the cystic duct to inject the CBD, which may become a difficult task in the case of intense inflammation/scarring (acute or chronic cholecystitis...), or anatomy variations, such as a short cystic duct or serrated Heister's valves. As well as failure, such difficulties may favour specific complications of this technique, such as CBD perforation and biliary leakage, increased rates of post-surgical procedures and overall complications[91]. If we add that, whenever IOC shows CBDS, patients eventually referred to postoperative ERC and ES may possibly be exposed to the risk of a third surgical procedure for CBD clearance if ERC is unsuccessful, the greatest advantage of IOC is seemingly within a totally

laparoscopic management of CBDS.

Laparoscopic US

Laparoscopic US (LUS)[28,92], whose rationale is that the effectiveness of US is maximal when the probe is placed nearer to the CBD, represents the most recent tool to diagnose CBDS intraoperatively. The latter technique is obviously less invasive than IOC, although specific instrumentation and ultrasonographic skills are needed. In expert hands, LUS is reported to have a sensitivity of 92%-95%, and a specificity of 99%-100% for detecting CBDS[28,92,93]. In specialized environments, LUS has become the primary routine imaging method for evaluating the bile duct during laparoscopic cholecystectomy, thus relegating IOC to being performed in only 4%-23% of cases[28,92], whenever LUS imaging is inadequate, stronger clinical indicators of choledocholithiasis are present, or biliary anatomy remains uncertain[92]. Moreover, LUS success rate is higher than that of IOC (95%-99% *vs* 92%-97%)[19,92,93] and operative times are shorter (5-8 min *vs* 15-16 min)[19,93]. Furthermore, LUS does not subject the patient to the risk of bile duct injury, and it may be performed for pregnant patients because it involves no ionizing radiation[92]. Finally, the only equipment required for the study is the ultrasound machine and probe, making routine LUS significantly cheaper (less 46%) than routine IOC[94]. The main limitations of LUS are the learning curve associated with its initial usage[92,94] and the inability of LUS to identify bile duct anatomy in some cases, where surgeons should preferably have a low threshold for performing IOC[92]. For all these reasons, LUS has not yet become popular worldwide and, even if it finally does, IOC will probably not be abandoned, as it provides some additional information in difficult cases.

MANAGEMENT

CBDS management consists of CBD clearance and may be accomplished by surgery (traditional and laparoscopic), endoscopy and lithotripsy[87,95-97]. Since, in most cases, CBDS are due to gallstone migration from the gallbladder which is still in situ, there is also a formal indication for cholecystectomy. Such a frequent eventuality may be dealt with in several ways, as a one-step or two-step-procedure, variously associating the above reported techniques and laparoscopic cholecystectomy.

A recent extensive literature review[98] of 16 published randomized trials comparing the results of cholecysto-choledochal management by open surgery, laparoscopy and various endoscopic-laparoscopic protocols, did not show any significant differences concerning overall mortality and morbidity, ranging from 0%-3% and 13%-20%, respectively. Nevertheless, the cumulative rate of retained stones was significantly lower after open surgery (6%) than after two-step protocols (including laparoscopic cholecystectomy and ERC + endoscopic sphincterotomy regardless of the sequence of procedures - 16%). Laparoscopic treatment was also associated to a lower rate of retained CBDS than post-cholecystectomy endoscopic management (ERC + ES) (9% *vs* 25%). Moreover, laparoscopic management resulted as being associated to shorter hospital stay and reduced in-hospital charges than sequential two-step management in some studies[96,99,100].

Although the analysis of recent literature seems to be in favour of a large-scale diffusion of one-stage laparoscopic management of cholecystocholedochal lithiasis, as witnessed by the encouragement to surgeons to train in laparoscopic CBD clearance by the British Society of Gastroenterology guidelines[38], nevertheless, in common practice, only 20%-21% of American surgeons regularly perform laparoscopic CBD exploration[100,101], and 75% consider preoperative ERC as the preferred approach[101]. The reasons for such a limited diffusion of laparoscopic CBD exploration are the necessary learning curve for a not-so-frequent procedure, the non-reproducibility of referral centers' results in elective patients[40], long operative times and lack of equipment[101].

Endoscopic procedures

ERC + ES: First introduced in 1974[102], ERC followed by endoscopic sphincterotomy has become the most widely used method for imaging and treating CBD stones[103,104]. The technique consists of the endoscopic identification of the papilla major (Vater papilla), its cannulation in order to perform ERC and endoscopic sphincterotomy followed by CBDS extraction by Dormia Basket or balloon. Endoscopic sphincterotomy success rate is reported to exceed 90%[22,87,88,105,106]. Although regarded widely as a safe procedure, large series have recently shown 5%-9.8% morbidity and 0.3% to 2.3% mortality[26,75-77,106,107], mostly due to postoperative acute pancreatitis, bleeding and perforation; the latter, reported in 0.3%-1% of cases, carries a mortality rate of 16%-18%[77,106,108,109].

Timing for ERC is also a matter of debate, as ERC may be performed before, after, or even during cholecystectomy, according to the so-called "rendez-vous technique".

Preoperative ERC[44,87,88,96] presents the drawback of needing a second surgical procedure to treat gallstones (cholecystectomy), but has the great advantage of allowing for a "strategy update" before surgery: if endoscopic sphincterotomy is successful, cholecystectomy will complete the mini-invasive management of cholecysto-choledochal lithiasis; if it is not, the "second step" will be a surgical procedure aimed at managing both gallstones and CBDS, which, in most environments, will be performed by laparotomy. The other major issue of this approach is patient selection to undergo ERC, since the systematic use of ERC is no longer acceptable in patients who are candidates for cholecystectomy (see above).

Performing ERC after cholecystectomy [22,31,75,110] is also a "two step management" of cholecysto-choledochal lithiasis, and has the great advantage of performing ERC in virtually only those cases really needing CBD clearance, thus reducing to a minimum any possible ERC-related complications and costs. Unfortunately, the main

drawback of postoperative ERC is the need for a third surgical procedure whenever postoperative ERC fails.

The rendez-vous technique[87,88,111,112] avoids some of the critical issues of other techniques, since it involves CBDS diagnosis and the synchronous management of both gallstones and CBDS during the same procedure ("one step"), although it needs the systematic availability of dedicated instrumentation and a second team to perform intraoperative ERC whenever IOC/laparoscopic US shows CBDS.

Endoscopic papillary balloon dilatation: Endoscopic papillary balloon dilatation (EPBD) was first introduced with the purpose of extracting CBDS minimizing the damage to the sphincter of Oddi[113]. Several studies have shown that EPBD alone or in combination with small sphincterotomy and lithotripsy can be used for the management of difficult biliary stones[114-118]. The rationale of performing a minimal sphincterotomy before proceeding with EPBD is that it can provide a larger opening and prevent perforation and bleeding, thus making this technique especially attractive in patients who are at risk of bleeding or in those with altered anatomy in whom a full sphincterotomy cannot be achieved. After minimal sphincterotomy, a guidewire is inserted into the bile duct and a balloon catheter is guided over the wire. The balloon is inflated until it reaches a diameter of 15-20 mm. Endoscopic papillary dilation is performed slowly (approximately 1 min of balloon dilation time)[119]. The results of a Japanese multicentric trial with a mean follow up of 6.7 years demonstrated that there is a lower risk of stone recurrence following EPBD when compared with ES[120]. Nevertheless, despite its premises of lower invasiveness, a recent meta-analysis[121] including 15 randomized trials comparing endoscopic sphincterotomy and EPBD showed this latter to be associated to a lower rate of stone removal (RR= 0.90), a more frequent need for mechanical lithotripsy (RR = 1.34), and a higher risk of pancreatitis (RR = 1.96). Somewhat confirming its not so low invasiveness, more recently EPBD has been associated with higher morbidity and severe complications than sphincterotomy, including acute pancreatitis and perforation[115]. The rate of these complications can be reduced by strict patient selection, avoidance of forced procedures, optimal dilation duration and immediate conversion to an alternative procedure if any difficulty is encountered during EPBD[115]. Conversely, EPBD is associated with reduced risk of bleeding and early/long term infections, thus making this option especially indicated in older patients, those who are at risk of infection and those who are affected by coagulopathy[122].

Surgical procedures

Open surgery: Until the late 1980s, gallstones were treated by open cholecystectomy and CBDS were managed by open CBD exploration and clearance, which was performed by duodenotomy and sphincterotomy or bilioenteric anastomosis[45,123].

Although open surgery is regarded nowadays as the last resource or obsolete therapy of CBDS, recent literature seems to show its superiority to ERC in achieving CBDS clearance[96,98], without increasing morbidity/mortality (20% *vs* 19% and 1% *vs* 3% for open surgery and ERC, respectively)[98]. An emerging issue regarding CBDS management and biliary surgery in general is that, while the treatment of gallstones is mostly performed mini-invasively by laparoscopy and/or endoscopy, open biliary surgery is performed increasingly less outside centers specialized in hepato-bilio-pancreatic surgery. Such a trend towards the "super-specialization" of surgeons, in accordance with centralization policies theoretically aimed to improve the quality of surgery and to reduce its costs, raises new issues regarding the most appropriate management of those patients, whose number is small but not negligible, presenting with complex cases or needing conversion/revision by open approach, with potentially disastrous consequences. The answer we will give to such a dilemma will definitely have an impact on the education of next generation's general surgeons.

Laparoscopy: Since 1991[21], CBD exploration may be performed laparoscopically. After Calot's triangle dissection, laparoscopic IOC and/or US are used in order to identify CBDS. Whenever CBDS are found, clearance is usually attempted by "water flush" by means of an irrigator. The latter procedure may be performed through the cystic duct, if it is adequately large, or through vertical choledochotomy. If this latter manoeuvre fails, choledochotomy may also allow for the introduction of a choledochoscope and CBDS retrieval by Dormia basket.

Laparoscopic CBD management, in expert hands, is reported to be at least as effective as ERC in clearing the CBD[22,75,98]. After clearance, in most cases choledochotomy is sutured without the need for any drainage (T-tube-Kehr -drain), whereas the latter may be required if the CBD is inflamed[32,124-126]. Finally, although the operating time is definitely longer than that needed to carry out a simple laparoscopic cholecystectomy, both gallbladder and CBDS are treated during the same intervention, thus avoiding a second hospitalization and procedure[22,96,100].

Conversely, laparoscopic exploration has particular drawbacks, which limit its diffusion outside specialized environments. CBD exploration/clearance needs costly instruments and adequate surgical skills. The feasibility of laparoscopic CBD exploration depends on several variables, including tissue status (inflammation, adhesions...), patient anatomy (length/size/insertion of cystic duct, size of CBD) and number/size/location of CBDS. Recently, our group proposed a "laparoscopy first" attitude in managing CBDS, involving a systematic laparoscopic exploration before deciding whether to proceed with CBD exploration or just cholecystectomy, in the latter case postponing CBDS management to postoperative ERC[32].

Some small series/case reports showed the feasibility of choledocho-jejunal anastomosis by laparoscopy for

CBDS[127,128]. Nevertheless, the difficulty of the procedure, requiring very experienced surgeons, and the long operating time (300-358 min)[127,128] make this technique a late option of CBDS management in very specialized environments.

Emerging mini-invasive surgical techniques: Recent advances in minimally invasive surgery, including single incision laparoscopy (SILS), natural orifice translumenal endoscopic surgery (NOTES), and robotics, to some extent show the possible future of biliary lithiasis management, although lesser dexterity on the part of surgeons, the need for dedicated devices/advanced skills and the intrinsically technical difficulty of CBD exploration limit the diffusion of these approaches to cholecystectomy at the moment.

Although SILS cholecystectomy has been increasingly reported[129-133], interestingly, very little information is reported regarding patient selection and CBD status. IOC has not been attempted systematically (5%-93%)[129-133], probably partly because of the difficult triangulation through the single access. When attempted, IOC had a success rate of 88%-93%[129,132,133]. Moreover, when IOC finally showed CBDS, only two of the relevant papers reported the accomplishment of laparoscopic CBD clearance[129,133]; of these, only Yeo[133] reports a single port CBD clearance (1 of 5 cases with CBDS), whereas Hawasli[129] "converted" the procedure to traditional laparoscopy. SILS is presently reserved for specially selected, non-obese patients undergoing cholecystectomy[133].

NOTES has also been used for cholecystectomy, in 92%-97% of cases with a transvaginal access and in 3%-8% of cases with a transgastric one[134,135]. Interestingly, neither CBD preoperative work up nor IOC are reported in most articles[134-136], whereas others considered the need for an intraoperative diagnosis as being an exclusion criteria for NOTES[137,138]. In the only series reporting the IOC technique, significantly only 33 out of 83 of eligible patients (40%) underwent transvaginal cholecystectomy[138], 13 transvaginal IOC and 3 laparoscopic CBD clearance requiring one additional 12-mm port[138]. NOTES cholecystectomy is performed in non-complicated cases in non-obese patients, who are almost exclusively women, since it is mostly performed transvaginally.

First proposed for cholecystectomy in 1994[139], robotics may theoretically offer both the advantages of mini-invasive approach and the dexterity of open surgery, which should be useful in particular during demanding procedures such as CBD exploration. Nevertheless, few papers report any manoeuvre/procedure to diagnose/treat CBDS. First described in 2004[140], small series of robotic laparoscopic exploration of CBD have recently been reported[141,142], showing a longer mean operating time (220 min *vs* 169 min) and a shorter median hospital stay (4 d *vs* 11 d) compared to open surgery[142]. These procedures involve the placing of five or more trocars. This technique may currently be considered at best an option for CBDS refractory to ERC[142] in order to avoid open surgery or percutaneous drainage in very specialized environments.

Single port robotics[143,144] intuitively present all the advantages of SILS and robotics. Unfortunately, switching from cholecystectomy to IOC (and potentially to CBD exploration) is not such an easy task to accomplish, owing to the difficult triangulation and the need to change instruments[144]. Single port robotic IOC involves another incision[143] and longer operating times[143,144], whereas CBD exploration has not yet been described by single port robotics.

Although papers reporting CBDS management by robotic choledocho-jejunostomy are extremely sporadic[145,146], the increasing evidence of an improved dexterity of the robotic hepatobiliary surgeon[147] seems to indicate a possible future management of complex cases.

Lithotripsy

First introduced in 1982[148], lithotripsy represents the theoretically ideal management of CBDS, as it may enable the clearing of the CBD without any interruption of the CBD wall or sphincterotomy. Although lithotripsy may technically fragmentize gallbladder stones too, it cannot be considered a radical treatment of synchronous cholecysto-choledochal lithiasis, as gallstone aetiology is considered as being due to lithogenic bile in the gallbladder (and therefore gallstones are destined to recur after lithotripsy). Moreover, since fragmentized gallstones are smaller, they may be supposed to have a higher risk of migration into the CBD, with choledocho-lithiasis recurrence. However, lithotripsy presents the advantage of being a one time (or few times) management of CBDS. For all these considerations, lithotripsy does not allow for avoiding cholecystectomy, thus becoming a very attractive option whenever cholecystectomy has already been performed or is not indicated. Reported to be performed in several ways (mechanical, electrohydraulic, laser, and extracorporeal shock wave)[23-25,149], lithotripsy also presents the drawback of needing dedicated instrumentation and skilled personnel, which are not always available, thus limiting its diffusion worldwide.

Endoscopic mechanical lithotripsy: endoscopic mechanical lithotripsy is usually performed after endoscopic sphincterotomy for CBDS that cannot be removed through a Dormia basket or balloon catheter. Introduced through the same scope utilized for ERC, lithotriptors consist of a large hard-wire basket with an additional metal spiral sheath, which is advanced over the basket into the CBD. When in position, the metal basket is opened to capture the stones and pulled back to the external hard-duct of the lithotriptor and lithotripsy is performed. The so-called "out of the scope" technique, where the endoscope is removed to introduce the Soehendra lithotriptor and crush the stones blindly[150,151], has been progressively replaced by the "through the scope" technique, where the lithotriptor is inserted in the endoscope itself and the whole procedure is performed under vision, reaching a

CBD clearance in 80%-90% of cases[149,151-153].

Conditions associated to failure of lithotripsy are CBDS size exceeding 3 cm[154], since stones could not be captured, and stone impaction in the CBD[155]. Basket impaction or rupture of the basket traction wire are complications unique to mechanical lithotripsy[24,156]. Some patients cannot tolerate the prolonged lithotripsy operating time, thus, the procedure has to be conducted in several sessions[154,155], and general anaesthesia is recommended in selected cases[157]. According to several authors, a stent should be placed for bile drainage if the CBD stones could not be removed in the first session of mechanical lithotripsy[158-160].

Endoscopic electrohydraulic lithotripsy: Endoscopic electrohydraulic lithotripsy, using a "baby-mother" scope system[161,162], may also be used for difficult CBDS. The mother scope has a large operating channel to accommodate the baby scope carrying the 4.5 Fr calibre probe with an electrohydraulic shock wave generator set at an output of 2000 V, generating high-frequency hydraulic pressure waves. Because these shock waves can also destroy normal tissues, it is important that the probe is placed close to the stone. The CBDS removal rate ranges from 74% to 98%[161,162]. However, this procedure needs costly and fragile endoscopes[97] as well as the excellent coordination of two very experienced endoscopists. The latter problem has been potentially solved by the introduction of the so-called SpyGlass system[163,164], where the same endoscopist controls both duodenoscope and cholangioscope (SpyScope). After ES, the SpyScope is introduced through the therapeutic duodenoscope into the biliary tree, where electrohydraulic lithotripsy can be used via a 3-Fr electrohydraulic lithotripsy probe.

Endoscopic laser lithotripsy: Since the mid-nineties, laser-induced shock wave lithotripsy has been used in shattering huge stones under fluoroscopic visualization, which was reqested because of the risk of heat-induced biliary perforation[165]. Nowadays, single-operator steerable cholangioscopy (SpyGlass) allows the safer use of laser lithotripsy under direct vision[166,167]. A 93%-97% removal rate of the bile duct stones has been reported, with mild complications in 4%-13% of cases[167-169]. Among the several types of laser lithotriptors developed, the holmium laser (holmium:yttrium aluminum garnet or Ho:YAG) is the newest one, but its use is still limited, also owing to the need for costly equipment[119]. Recent laser lithotriptors combine the advantages of dye and solid-state lasers at a reasonably low price[166], thus potentially allowing for a future progressive diffusion of laser lithotripsy worldwide[166,167].

Following the encouraging results of laser lithotripsy by endoscopy, laser lithotripsy has recently been proposed during laparoscopy[170], open surgery[171], or percutaneously[172].

Extracorporeal shock-wave lithotripsy: External shock wave using extracorporeal shock wave lithotripsy (ESWL) is also used for difficult CBDS under ultrasound control or fluoroscopic guidance, after nasobiliary tube placement for contrast instillation [97,173]. In the case of first generation lithotriptors, the patient needed to be immersed in a water bath, whereas modern lithotriptors employ water-filled compressible bags. General anaesthesia is usually needed as the discomfort produced may not be adequately controlled by conscious sedation. The critical determining factors for success of ESWL are stone size/structure[174] and CBD diameter[175]. ESWL has particular contraindications, such as portal thrombosis and varices of the umbilical plexus, and may be associated with adverse events such as transient biliary colic, subcutaneous ecchymosis, cardiac arrhythmia, self-limited haemobilia, cholangitis, ileus, pancreatitis[176,177], and, more rarely, biliary obstruction, bowel perforation, lung injury and splenic rupture[174]. Multiple ESWL sessions may be required. The recurrence rate of CBD stones during a 1 to 2 year follow up is around 14%[178]. In a randomized trial comparing fluoroscopy-guided ESWL and laser lithotripsy, the latter is preferable for a successful stone free rate (97% *vs* 73%)[173]. Therefore, ESWL is at present not considered as being the first line treatment of difficult bile duct stones[97].

PARTICULAR SITUATIONS

Concomitant complication

Acute cholecystitis: Whether acute cholecystitis is associated with CBDS is a matter of debate, as some authors have found CBDS in 9.1%-16.5% of patients presenting with acute cholecystitis (*vs* 6.6%-7.7% of those with uncomplicated lithiasis[179,180]), while others did not[181]; we ourselves found acute cholecystitis to be related to the absence of CBDS, thus entering in our score system[44].

Diagnosis of CBDS may be difficult in the presence of acute cholecystitis, where various alterations in liver serology and hyperpyrexia may be observed, thus possibly "hiding" CBDS. Moreover, since the effectiveness of US and CT is suboptimal in diagnosing CBDS, they can pass undiagnosed. Recently, the systematic use of MRC has been proposed for the diagnosis of acute cholecystitis, coexistent choledocholithiasis, and possible complications such as gangrene and perforation in patients with inconclusive clinical, laboratory, and sonographic findings[182,183].

Since acute cholecystitis may include a wide variety of clinical pictures, ranging from asymptomatic cases presenting merely the doubling of the gallbladder wall at US, to those with abdominal pain/tenderness suitable for conservative treatment, or with life-threatening conditions needing emergency surgery, the management of synchronous acute cholecystitis and CBDS should be tailored considering gallbladder status and the patient's general conditions. In general, the presence of acute cholecystitis may increase the difficulty of dissecting Calot's triangle by laparoscopy, as well as of cannulating the cystic duct to perform IOC or proceed with CBD laparoscopic exploration. Therefore, CBDS associated with acute cholecystitis are gener-

ally considered an indication for ERC[184], as confirmed by a recent survey of 859,747 patients who underwent emergency and/or urgent laparoscopic cholecystectomy and where IOC was performed in only 29% of cases and CBD exploration in no more than 1%[185]. Nevertheless, the laparoscopic management of acute cholecystitis and CBDS is feasible, with an acceptable 12% conversion rate and 29% morbidity[186]. As we have already mentioned[32], a laparoscopy-first attitude, intraoperatively evaluating the real feasibility of laparoscopic CBD exploration, may allow for CBD clearance in roughly 2/3 of cases, thus reducing the number of postoperative ERCs to a minimum, without excessively long/risky procedures and inopportune conversions.

Acute cholangitis: Biliary decompression is considered as being the primary treatment of gallstone-related acute cholangitis. As acute cholangitis may have various clinical presentations, elective biliary decompression may be planned by endoscopy or laparoscopy in mild to moderate disease, whereas emergency endoscopic sphincterotomy has to be performed in the case of severe sepsis not responding to antibiotic therapy[29,38]. Any surgical treatment in these patients is associated with higher mortality than for endoscopy and therefore should be avoided[187,188]. Given the potentially life-threatening condition, significantly, even if ERC does not show any CBDS, there is a formal indication for endoscopic sphincterotomy followed by balloon or basket trawl of the CBD[38].

Acute pancreatitis: The management of biliary acute pancreatitis has evolved over the last 30 years. Following the results of randomized studies[189,190], the United Kingdom guidelines for the management of acute pancreatitis advocated urgent therapeutic ERC in every patient with acute pancreatitis by suspected gallstone etiology[191]. With the advent of EUS and MRC[192], this management policy has been modified, according to the principle that every effort should be made to identify biliary obstruction before resorting to ERC[193]. Lastly, the American Gastroenterological Association Institute guidelines on acute pancreatitis recommend that early ERC is not indicated in patients with predicted severe pancreatitis without concomitant cholangitis or high suspicion of CBDS[194].

Elderly, poor conditions and anticoagulant/antiaggregant therapy

The aging population of Western countries together with the increasingly less invasive nature of CBDS management is rekindling the debate concerning the most appropriate treatment not only after the age of 65 (elderly) but also after the age of 80 (very elderly), where procedure-related complications may become severe or fatal.

Although the small size and retrospective nature of the studies do not confer a clear evidence, no clear benefit of any technique has been shown over another in this class of patients. ERC and endoscopic sphincterotomy are proposed for CBDS retrieval in the very elderly[157,195-197]. Because elderly patients often receive anticoagulant/antiplatelet agents for underlying heart and cerebrovascular diseases, the risk of bleeding after endoscopic sphincterotomy is higher in these patients[77,198] and the ASGE guideline has recommended discontinuing anticoagulant agents before ES[199]. Moreover, patients who are old[200] and/or have haemostasis problems (such as cirrhosis)[201] have been recommended for management by endoscopic balloon dilation instead of ERC, with similar results to those of younger patients (apart from a higher number of endoscopic sessions)[200]. In very old patients and those with serious co-morbidities, where other endoscopic or surgical procedures may confer unacceptably high risks, endoscopic biliary stenting is a useful alternative[202].

Similarly to sequential treatment (ERC followed by delayed laparoscopic cholecystectomy)[203], the rendezvous technique has shown longer operating times and hospital stays in octogenarians compared to younger patients[112], whereas the two approaches did not show significant differences from each other among the octogenarian population[112]. In general, although results of mini-invasive CBDS management in the very elderly patients are seemingly worse than in younger patients, they mostly consist of longer operating times and hospital stays; since clinical impact and morbidity are seemingly lower than those of traditional surgery, age should not contraindicate *per se* CBDS management.

Biliary stent placement is a possible alternative in elderly patients[158,204], but is likely to put the patients at risk for cholangitis as a result of stent clogging. To avoid this adverse event, a periodic (*e.g.*, every 3 mo) exchange of biliary stents is needed, which makes this option far less attractive whenever any other technique is feasible.

An emerging dilemma is whether to proceed with cholecystectomy after successful CBD clearance in patients of 80 years old or older, where laparoscopic cholecystectomy may be presumed to be associated to increased morbidity/mortality. Indeed, a review[203] reporting the results of laparoscopic cholecystectomy in the very elderly showed a cumulative mortality rate of 2% in octogenarians, but this value dropped to virtually 0 if patients presenting with acute cholecystitis and/or concomitant severe disease were excluded, to some extent suggesting not postponing elective laparoscopic cholecystectomy in fit octogenarians.

Technical difficulty

The wide variety of treatment options for CBDS available nowadays makes it impossible to draw up any algorithm/protocol including any possible challenging situations for endoscopists and surgeons. Any systematic approach to CBDS by a single technique, as has been proposed for decades by authors having a great expertise in only one field (endoscopy, laparoscopy, lithotripsy), is slowly but progressively giving way to a more patient-tailored approach, where the pros and cons of different approaches are taken into account[31,33]. Obviously, the in-

creasing availability of instruments and skills in the same environment will allow for a diffusion of such a multidisciplinary approach to CBDS. Moreover, as already pointed out, it is nowadays suggested that asymptomatic CBDS should be managed more conservatively, as some will pass through the papilla within a few weeks of cholecystectomy[31,35]. Thus, following this more multidisciplinary and cautious policy, an unsuccessful laparoscopic CBD exploration, for example, is increasingly often considered an indication not for conversion to open surgery and transduodenal CBD clearance or biliodigestive anastomosis, but rather for a postoperative conservative or mini-invasive approach by ERC[32], based on the assumption that a difficult laparoscopic CBD clearance does not necessarily mean a difficult endoscopic management.

Postcholecystectomy CBDS

Postcholecystectomy CBDS may be due to residual CBDS misdiagnosed at surgery, as happens after up to 2% of cholecystectomies performed without cholangiogram[205], or recurrent CBDS. The latter are due to intrahepatic or intraCBD lithiasis and are therefore not treated in this review. Other than the already described clinical pictures, residual CBDS are the most frequent cause of a "postcholecystectomy syndrome", including symptoms originally attributed to gallstones, such as abdominal pain, nausea, vomiting, dyspepsia, and persistent biliary colic and liver serology alterations[206].

Diagnosis of CBDS after cholecystectomy may be difficult, as the absence of a gallbladder may lead to the exclusion of a biliary origin of aspecific symptoms/laboratory test alterations, thus causing a delay in diagnosis. Moreover, transabdominal US is less accurate in postcholecystectomy patients, since the CBD diameter may be larger because of cholecystectomy or old age of patients[55,207,208], where the upper limit for a "normal" CBD diameter is raised to 7.6-10 mm[207-209]. Since the total number of patients undergoing second-line examinations for residual CBDS and finally presenting this feature is 33%-43%[208,210], systematic ERC should result as being unnecessary in more than half of the patients and is therefore seemingly not indicated as a second-line examination. Among less invasive imaging techniques, although MRC and EUS have shown similar results in this setting[208,210], the need for ERC in 1/3 of patients or more should prompt us to favour EUS, possibly followed by ERC and endoscopic sphincterotomy during the same session, for a theoretical one-step-management of CBDS.

The absence of gallbladder and the consequent possibility to avoid surgery and general anaesthesia make endoscopy the most attractive option for several authors[211,212]. For the same reason, lithotripsy may intuitively be useful in this setting[213], whereas open surgery is the last option after the failure of mini-invasive approaches[214]. Although laparoscopic CBD clearance is also possible in such a context in expert hands[215], such a procedure is likely to result as being more difficult owing to the sequelae of cholecystectomy (adhesions, scarring), thus requiring advanced laparoscopic skills.

CONCLUSION

CBDS diagnosis and management can nowadays rely on various imaging techniques and mini-invasive treatments, including endoscopy and laparoscopy. Concerning diagnosis, IOC, EUS and MRC have similar results and very low morbidity and may be used indiscriminately. There is no consensus regarding the best management of CBDS, where surgery has seemingly slightly better results and a lower pancreatitis rate, which are counterbalanced by the need for specific instrumentation and advanced laparoscopic skills. In this regard, significantly, the British Society of Gastroenterologists encourage surgeons to train in laparoscopic CBD exploration[38] while, conversely, laparoscopic surgeons mostly prefer ERC to treat CBDS[101]. According to recent guidelines, the management of CBDS will be increasingly tailored not only to a specific patient but also to the available resources of a specific environment in order to have the best possible management. The worldwide diffusion of mini-invasive management (by laparoscopy/endoscopy) of biliary disease goes in the opposite direction of the recent trend of the "centralization" of open biliary surgery, which is being performed increasingly less outside specialized centers. Such considerations raise new issues regarding the most appropriate management of complex cases needing conversion/exploration by an open approach, as the new generation of laparoscopic surgeons may not be able to deal with their own complications.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (15): Laparoscopic resection of gastrointestinal

Laparoscopic distal pancreatectomy for adenocarcinoma of the pancreas

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Abstract

Since the first report on laparoscopic distal pancreatectomy (LDP) appeared in the 1990s, the procedure has been performed increasingly frequently to treat both benign and malignant lesions of the pancreas. Many earlier publications have shown LDP to be a good alternative to open distal pancreatectomy for benign lesions, although this has never been studied in a prospective, randomized manner. The evidence for the use of LDP to treat adenocarcinoma of the pancreas is not as well established. The purpose of this review is to evaluate the current evidence for LDP in cases of pancreatic adenocarcinoma. We conducted a review of English language publications reporting LDP results between 1990 and 2013. All studies reporting results in patients with histologically proven pancreatic adenocarcinoma were included. Thirty-nine publications were found and included in the results for a total of 309 cases of pancreatic adenocarcinoma (potential double publications were not eliminated). Most LDP procedures are performed in selected cases and generally involve smaller tumors than open distal pancreatectomy (ODP) procedures. Some of the papers report unselected cases and include procedures on larger tumors. The number of lymph nodes harvested using LDP is comparable to the number obtained with ODP, as is the frequency of R0 resections. Current data suggest that similar short term oncological results can be obtained using LDP as those obtained using ODP.

Key words: Adenocarcinoma of the pancreas; Laparoscopy; Distal pancreatectomy; Surgical margins; Pancreatic resection

Core tip: There are about 300 published cases of laparoscopic distal pancreatectomy for adenocarcinoma reported in the English literature. None of these cases has been included in randomized prospective work and it is doubtful that such a study will ever be conducted. This objective of this review was to evaluate the appropriateness of laparoscopic distal pancreatectomy as treatment for pancreatic adenocarcinoma. The results suggest that this minimally invasive technique may be safely applied to treat the disease in addition to the other more established indications for the operation.

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BACKGROUND

In the late 80s and early 90s, laparoscopy became an increasingly common surgery. Laparoscopic cholecystectomy, followed by adrenalectomy, splenectomy, and appendectomy, became standard procedures[1-6]. Today,

laparoscopic resections for colon cancer are considered safe and oncologically equivalent to open surgery[7]. In fact, the introduction of laparoscopy may be described as the largest uncontrolled, unrandomized surgical trial ever undertaken.

In cases of pancreatic malignancies, the initial use of laparoscopy was for staging and palliative procedures[8-11].

In 1994, a study introduced the possibility of laparoscopic distal pancreatectomy (LDP) and assessed the procedure in a porcine model; the authors concluded that the procedure should be possible in humans[12]. In that same year, Gagner performed a laparoscopic Whipple to treat a patient with chronic pancreatitis[13].

Only two years later, Gagner *et al*[14] reported retrospectively laparoscopic distal pancreatectomy in eight patients with presumed benign tumors (and one unclear) as well as four enucleations. However, this was also the first report on LDP in a patient with adenocarcinoma, as one of the tumors was a cystadenocarcinoma. Cuschieri *et al*[15] reported results of the procedure performed in five patients to treat chronic pancreatitis, and, in the same year, case reports further supporting this new approach were published[15,16]. The procedure was still in its infancy and was reserved for indications other than pancreatic adenocarcinoma[17,18].

In 1997, the first report on laparoscopic distal pancreatectomy from the United States was published[19]. In the same year, the first case report on the preservation of the spleen was published[20].

The second report that included adenocarcinoma of the pancreas reported 2 LDP procedures, one of which was to treat adenocarcinoma[21].

Spleen preservation was further established in the literature in 1999[22,23]. The higher rate of spleen preservation seen using LPD compared to open distal pancreatectomy (ODP) has generally been considered positive, while resection of the splenic vessels adds to the oncological radicality of the operation and may increase the number of lymph nodes retrieved[24].

In a series of 15 patients who underwent LDP between 1993 and 2000, two were found to have adenocarcinoma of the pancreas. However, the margin status and lymph node number for these patients is unknown[25].

Fernández-Cruz *et al*[26] reported on the results of LDP procedures used to treat chronic pancreatitis in 2002 and compared those results to those of open surgery. This small study of 5 patients suggested that LDP was a feasible alternative for patients with this disease.

In a series of 6 attempted (5 successful) LDPs from 2002, two of the patients underwent operations for adenocarcinoma[27]. One underwent an operation with a curative intention and was reported alive and free of tumor at the 2-year follow up. The other patient underwent the operation as part of palliative treatment, and a liver metastasis was removed during the same operation. For these two patients, the number of lymph nodes resected was reported as 19 and 6, respectively, and the margin statuses were R0 and Rx, respectively. Although this report did not compare LDP to ODP, it was the first to report oncological findings.

When the use of LDP was reviewed in 2003, a total of 47 cases were found, as reported in 18 articles. In only 3 of those cases did patients have adenocarcinomas, and the indication for LDP was still found to be non-malignant lesions[14,21,28]. At the same time, the indications were evolving from generally including neuroendocrine pancreatic tumors and pancreatitis to also including cystic neoplasias of the pancreas[29,30].

Early results of procedures performed between 1997 and 2003 included a retrospective analysis of 12 LDP procedures that included 4 adenocarcinomas. However, the lymph node count and margin status were not mentioned[31]. In another report from 2004, 17 LDPs performed between 1997 and 2002 were presented. Four patients in that study had adenocarcinoma (3 cases were successfully resected with LDP) and two had positive surgical margins. The margin status for the entire cohort of patients with pancreatic lesions was 88% R0; the lymph node count was not provided, and the median survival was 12-13 mo[32].

In 2005, a large retrospective European multicenter study summarized the experience of laparoscopic distal pancreatectomy between 1995 and 2002, reporting that out of 127 patients, only 6 had pancreatic adenocarcinomas[33]. In the same year, a report on 21 prospectively registered patients undergoing LDP, including 3 adenocarcinomas, suggested the feasibility of LDP with a low morbidity rate; follow up was between 2 mo and 3.8 years and all patients were reported to be disease free[34]. All patients had an R0 resection and a median number of 18 lymph nodes were retrieved.

In a retrospective analysis of prospectively sampled data of 16 hand-assisted LDP procedures performed between 2002 and 2004, two LDPs were performed for adenocarcinomas that were either suspected or had been confirmed by fine needle aspiration. Final pathology showed one case of chronic pancreatitis and one of adenosquamous carcinoma. The R0 frequency was found to be 76% (calculation based on 13 patients with neoplasia in the pancreas) and the mean number of lymph nodes resected was 5.5[35]. In another article published in 2006, 15 cases of attempted LDP resulted in 3 conversions to ODP. These 3 surgeries were the only cases of pancreatic adenocarcinoma in the LDP group; the lymph node count and margin status were not described[36]. In a report on 13 LDPs performed to treat benign or low-grade malignant lesions, 3 adenocarcinomas were found during final pathology testing, but the lymph node count and surgical margins were not disclosed[37].

In 2007, a single-center analysis of 82 LDPs performed between 1998 and 2007, including 13 ductal adenocarcinomas, was presented with 90% R0 resections in that subgroup. Five other adenocarcinomas were included as well and all patients had R0 resections; the median survival for ductal adenocarcinoma was 14 mo[38]. In this report, suspected and overt malignant lesions were

operated on according to the principle of Radical Antigrade Modular PancreatoSplenectomy (RAMPS). Three of the operations were converted to open surgery and all included extended en block resection of other organs. In median, 14.5 lymph nodes were resected and the median tumor size was 5.3 cm.

In a single institution the retrospective analyses 58 of LDPs performed during the period of 1999 to 2005 were described. The group included 5 ductal adenocarcinomas as well as two mucinous cystadenocarcinomas; however, the surgical margin status or lymph node harvesting was unfortunately not mentioned[39]. The surgical approach was a retrograde dissection of the pancreas, and all of the patients with malignant diagnosis, except one who died 3 mo after surgery, were reported to be alive and disease free after a median of 26 mo follow up.

Another single institution report describes 22 LDPs, with 2 of the patients having malignant foci in the MCNs; both had R0 resection, but their lymph node status was not described[40].

The first multicenter experience from the United States was reported in 2008 (data from 2002 to 2006). In 8 centers (of which 3 were defined as high volume, > 30 LDP), 159 LDPs were attempted with 20 (13%) converted to open surgery. Of those, 16 were shown to have adenocarcinoma[41]. In a retrospective, case-matched study, LDP (n = 31) was shown to be equivalent to ODP (n = 62) with respect to complications, the operation time and bleeding, but the hospital stay was shorter in the LDP group. There were, however, no ductal adenocarcinomas in the group[42].

An early 10-year report (1996 to 2006) of 46 attempted LDPs in 5 institutions reports 9 ductal adenocarcinomas (as well as 1 mucinous cystadenocarcinoma). Twelve patients were converted to open surgery (including 4 ductal adenocarcinomas). All malignant cases completed with LDP had negative surgical margins[43].

In a retrospective analysis of 128 patients operated on for benign lesions of the pancreatic body or tail, in which patients were allowed to choose either LDP or ODP, 93 patients chose LDP and 35 ODP[44]. The results were similar, but the hospital stay was significantly shorter in the LDP group and splenic preservation was also more common in the LDP group.

In another article from 2008, 25 LDP and seven hand-assisted LDP cases were reported. The mean tumor size was 2.7 cm and 3 adenocarcinomas were included[45].

Twenty-five successful LDPs (31 attempted) that were prospectively evaluated were described; one patient may have had adenocarcinoma although this cannot be confirmed because other laparoscopic pancreatic procedures were described in the article as well. However, all of the patients with malignant diagnosis operated upon had negative surgical margins. Lymph node numbers were not given[46].

During the first 12-14 years after the introduction of LDP, approximately 50 cases of adenocarcinoma of the pancreas had emerged among the benign and low-malignant diagnoses that were considered established indications for LDP. Unfortunately, no randomized study has been performed on the applicability of LDP for adenocarcinoma of the pancreas. Tables 1 and 2 summarize published series on LDP that include patients with adenocarcinoma of the pancreas.

Table 1 Series on laparoscopic distal pancreatectomy that include adenocarcinoma of the pancreas and oncological outcome *n* (%)

Ref.	Number		Lymph nodes	R0	Tumor size (mm)
	Total	AC			
Song *et al*[47]	359	34 (9)	10.3[1]	92%	30 (26)[1]
Fernández-Cruz *et al*[38]	82	18 (22)	14.5[2]	90%[2]	53
Marangos *et al*[48]	30	28 (93)	5	93%	50
Taylor *et al*[43]	46	10 (22)	-	100%[3]	-
Melotti *et al*[39]	58	7 (12)	13	100%	35[4]
Asbun *et al*[49]	29	5 (17)	14 (19)[1]	97%	-
Edwin *et al*[32]	17	4 (24)	-	88 (50)[1]	28
Dulucq *et al*[34]	21	3 (14)	18	100%	42
Bärlehner *et al*[27]	5	2 (40)	19/6	R0/Rx	-
Sa Cunha *et al*[46]	31	1 (3)	-	100%[5]	37
D'Angelica *et al*[35]	16	1 (6)	5.5	77%	40

[1]Indicates numbers from adenocarcinoma diagnosis only; [2]Indicates numbers from ductal adenocarcinoma only (n = 13); the other 5 had R0 resection; [3]In 7 malignant cases completed with laparoscopic distal pancreatectomy, all having R0 resection; [4]Indicates numbers from ductal adenocarcinoma only, for 2 mucinous cystadenocarcinoma the corresponding number is 38 mm; [5]One ductal adenocarcinoma with R0 margins. AC: Adenocarcinoma of the pancreas.

This review was conducted to try to answer the question of whether LDP is a viable treatment option for adenocarcinoma of the body and tail of the pancreas.

GENERAL CONSIDERATIONS

Although LDP has not been compared to open distal pancreatectomy in a randomized prospective manner, there is a substantial literature supporting its use for benign lesions as LDP seems to result in less bleeding, a shorter hospital stay and reduced morbidity than ODP[47]. During the introduction of the laparoscopic technique, cost considerations have been raised because of the additional cost of the single use of surgical material. However, when the treatment cost is summarized, LPD seems to be at least as cost effective as OPD, mainly due to the shorter hospital stay[48-50].

Despite this, currently only approximately 20% of distal pancreatectomy (DP) cases in the United States are performed laparoscopically, which makes the results presented in the literature somewhat harder to extrapolate[51].

One of the benefits of LDP shown in a number of publications is the higher rate of spleen preservation seen compared to ODP. There are two methods for resecting the distal pancreas without resecting the spleen. One described by Warshaw, where the splenic vessels are removed and the spleen is left with circulation from the short gastric vessels and another where the splenic vessels are dissected from the back-side of the pancreas

Table 2 Series on laparoscopic distal pancreatectomy that include adenocarcinoma of the pancreas but not oncological outcome *n* (%)

Ref.	Number		Comment
	Total	AC	
Weber *et al*[50]	219	16 (7)	
Mabrut *et al*[33]	127	6 (5)	
Giulianotti *et al*[51]	46	8 (17)	Robot-assisted LDP
Butturini *et al*[52]	43	1 (2)	
Laxa *et al*[45]	32	3 (9)	7 hand-assisted
Corcione *et al*[37]	13	3 (16)	
Patterson *et al*[25]	15	2 (13)	
Lebedyev *et al*[31]	12	4 (33)	
Gagner *et al*[14]	8	1 (13)	
Santoro *et al*[21]	2	1 (50)	

AC: Adenocarcinoma of the pancreas; LDP: Laparoscopic distal pancreatectomy.

thus leaving the spleen with its circulation intact[52]. This, however, is not a valid argument for LDP in the setting of adenocarcinoma of the pancreas, as the oncological completeness of the operation is jeopardized when the spleen is spared[24,53,54].

ONCOLOGICAL CONSIDERATIONS

As there are no large reports on the long-term oncological results from using LDP and, as the cases published are likely to be highly selective, the oncological results cannot be accurately assessed. Surgical margins and the number of resected lymph nodes may serve as an approximation of the oncological outcomes. On the other hand, it should be kept in mind that LDP has mainly been performed for lesions that are presumed to be benign and, therefore, it is quite possible that the resections have been less extensive for this reason.

To be able to make a meaningful comparison, it is crucial to establish the true quality of ODP. Clean surgical margins are of paramount importance when dealing with adenocarcinoma of the pancreas and the rate of R0 resections with ODP is approximately 90% although numbers as low as 77% have been reported in large studies[53-57]. The number of lymph nodes harvested also varies, but the number is typically between 9-15[53-55,57].

The first comparative analysis that includes some oncological markers is from a prospectively registered database where 27 completed LDPs (28 attempted) were compared to 85 ODPs and found to be similar with regards to the operation time and complications. However, the hospital stay was shorter and blood loss lower after LDP. There was one case of ductal adenocarcinoma in the LDP group[58].

In 2009, a report on 148 distal pancreatectomies performed during the period of 2002 to 2007 showed that 50 were attempted LDPs (6 converted to open surgery) and 98 were ODPs. The LDP group included 6 adenocarcinomas. The complication rate was similar, but the hospital stay and blood loss was lower in the LDP group[59].

In a multi-institutional report on 219 LDP from 2009, 16 adenocarcinomas were found, and the study did not find any correlation between malignancy and an increased risk of major complications. However, the oncological completeness of the resections was not demonstrated[60].

In a retrospective analysis of 100 LDPs compared to matched 100 ODPs, the LDP group was found to have less blood loss and a shorter hospital stay. There were 17 cases of pancreatic adenocarcinoma in the LDP group[61]. Seventy-one completed (95 attempted) LDPs, including 3 ductal adenocarcinoma cases, were compared to 168 ODPs and found to result in less intraoperative bleeding and a shorter hospital stay. The number of lymph nodes harvested was similar, and positive surgical margins were significantly more common in the ODP group[62].

In 2010, a multicenter analysis comparing LDP to ODP in the settings of ductal adenocarcinoma at 9 US centers during the period of 2000 to 2008 was presented[55]. The study included a total of 212 patients operated on for ductal adenocarcinoma of the pancreatic body or tail. Of these, 23 (11%) were operated with LDP (4 converted to open procedure) and the remaining 189 with ODP. The number of lymph nodes examined and number of patients with positive surgical margins did not differ in the comparison (whole material and 3:1 matched). The hospital stay was shorter in the LDP group however. The median survival was 16 mo in both groups.

In a material of 343 distal pancreatectomies, performed during a 7-year period (2003-2009), 107 were attempted LDP (33 converted) and 97% of the patients in the LDP group had negative margins; also, the number of harvested lymph nodes was 6 compared to 5 in the ODP group. The number of cancer cases in the LDP group was 17, but it is not stated how many of those cases were adenocarcinomas of the pancreas[63].

In a description of the 10 year experience of DP for ductal adenocarcinoma of the pancreas performed between 1999 and 2008, a total of 50 patients were operated on, but 18 were excluded from the analysis as extended resections were performed[64]. Five were operated with LDP (starting in 2007, robot assisted). In the ODP group (n = 27), 87% had R0 resections and the median number of lymph nodes retrieved was 11.3 compared to 100% (median 8.2) in the LDP group. However, tumor size was 2.4 cm in the LDP group and 3.9 cm in the ODP; the median survival was 28 mo and did not differ between the groups. The authors conclude that LDP can be applied to selected patients with ductal adenocarcinoma of the distal pancreas.

Twenty-two LDP were compared to matched 22 ODP. In the LDP group there were 2 cases of malignant cystic tumors (that may represent mucinous cystic adenocarcinoma). The median tumor size in the LDP group was 2 cm compared to 5 cm in the ODP group. The number of lymph nodes and surgical margin status were omitted from the publication[65].

An interesting addition to the laparoscopic technique

is robot-assisted laparoscopy. This was first reported for LDP in 46 patients of whom 8 suffered from adenocarcinoma of the pancreas in 2010. Unfortunately, the number of lymph nodes retrieved and the margin status for this group is not mentioned in the publication; the median survival of patients who could be followed was 15 mo[66]. In another series on 22 ODP, 18 LDP and 17 robot-assisted LDP 2 adenocarcinomas were found in the LDP group and 11 in the ODP group. The mean lesion size in the ODP group was 5 cm compared to 3 cm in the LDP group. Fourteen lymph nodes were harvested in the ODP group, and in the LDP group, the corresponding number was 11. Two patients in the ODP group had positive margins against none in the LDP group[67].

Currently, the largest single center study of LDP is that of the Asian Medical Center, which presented data from 359 patients who underwent LDP between 2005 and 2010. Of those, 24 had ductal adenocarcinoma of the pancreas (in addition, 10 other adenocarcinomas were found), and this was the first report to clearly state that patients were operated on in accordance with the RAMPS procedure. The median number of lymph nodes harvested (from patients in the adenocarcinoma subgroup) was 10.3, and 92% were R0 resections. After a median follow-up of 10 mo, a median survival time was not determined, but the 1 and 2 year survival rates were reported to be 85%[68].

In 2011, a series of 29 patients who underwent LDP in a "clockwise" manner, starting with mobilization of the left colon flexure and continuing along the lower border of the pancreas, was published. In the 22 specimens where lymph node count was given, the median number of lymph nodes was 14; for the 5 patients with adenocarcinoma, the corresponding number was 19. The frequency of R0 resections for operations completed with laparoscopy was 100%, but one patient's procedure was converted to hand-assisted laparoscopy, which showed a positive surgical margin. The authors conclude that this technique shares similarities with RAMPS, as it allows for wide exposure of the pancreas and makes it feasible to include the left adrenal gland in the resection if needed (as was done in 3 of the cases presented)[69].

In a retrospective (prospectively registered) evaluation of 118 distal pancreatectomies in Toronto, 42 were attempted laparoscopically with 5 converted to open surgery. Hospital stay was shorter in the LDP group than the ODP group, but pancreatic fistula was more common in the LDP group; however, there were more grade B and higher fistulas in the ODP group. Two patients in the LDP group were found to have malignant masses (origin not described)[49].

In a 1:1 case-matched study of 60 patients (who were matched for final pathology and tumor size), hospital stay and intraoperative bleeding were more favorable in patients who underwent LDP when compared to ODP. Both groups had 7 patients with adenocarcinoma and the number of lymph nodes did not differ significantly, although there was a trend towards fewer lymph nodes being harvested in the LDP group[70].

In a retrospective analysis of 51 distal pancreatectomies performed over a period of 6 years, 35 were LDP and 16 were ODP. There were 4 adenocarcinomas in the LDP group compared to 3 in the ODP group, and one patient in each group had positive surgical margins. The number of retrieved lymph nodes was not discussed[48].

The second largest single center report of LDP for malignancy is of 21 patients with typical ductal adenocarcinomas of the pancreas as well as 7 other adenocarcinomas. The R0 frequency was 93%, but it should be noted that the tumors were larger (median size 5 cm) than in most other series and a significant proportion of operations included resections of organs other than the pancreas. The median number of lymph nodes investigated was 5, and 3 year survival was 30%[71].

A report on 122 LDPs performed over 10 years and focused on the changes between the first 66 and later 66 operations reported 18 malignancies (11 ductal adenocarcinomas) and 5 positive surgical margins (in results cover all 122 patients). Unfortunately, the number of lymph nodes resected was not stated[72].

In an Italian publication on 43 cases of LDP, which included 1 pancreatic adenocarcinoma, the median tumor size was 25 mm, and neither lymph node number nor margin status were mentioned[73].

In a publication focusing on LDP in cases of suspected malignancy, results from 12 LDP procedures were presented. Final pathology revealed 8 malignant cases, all with negative surgical margins, and the median number of harvested lymph nodes was 8. Four of the operations were hand-assisted and one was converted to open surgery[74].

Recently, the experience of a single center making the transition from using mainly open surgery to a laparoscopic approach to the distal pancreas was described. Between 2005 and 2011, 172 patients underwent operations, with 82 LDPs and 90 ODPs. During the first half of the period, all but one of the operations was ODP, but during the latter half, 83% were LDP. In the LDP group, 18 patients had pancreatic adenocarcinoma compared to 21 in the ODP group. No difference was found in lymph node retrieval or in R0 frequency, but hospital stay and intraoperative bleeding differed in favor of the LDP group[75].

In another recent publication focusing on the role of LDP to treat adenocarcinoma, no differences were found between LDP and ODP with respect to negative surgical margins (86% *vs* 88%) or lymph node clearance (11 *vs* 12) in 28 LDP and 32 ODP procedures. The median survival for the cohort was found to be 19 mo, and there was no difference between the two operation methods[57].

Similar results were presented in a study of 8 LDP patients compared to 14 who underwent ODP, which reported that 88% of LDP and 86% of ODP cases had negative margins. In this study, the number of lymph nodes was 16 (LDP) compared to 14 (ODP); 3-year survival was 82% in the LDP group compared to 74% (NS)

Table 3 Studies comparing laparoscopic distal pancreatectomy and open distal pancreatectomy that include adenocarcinoma of the pancreas and oncological outcomes *n* (%)

Ref.	Number		Number AC		Lymph nodes		Tumor size (mm)		R0	
	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP
Jayaraman *et al*[68]	107	236	17 (16)	47 (20)	6	7 NS	30	30	97%	96%
Vijan *et al*[66]	100	100	17 (17)	19 (19)	NS	NS	33	40	100%	100%
Stauffer *et al*[75]	82	90	18 (22)	21 (23)	16.5	11 NS	20	28	97%	94%
DiNorcia *et al*[67]	71	168	3 (4)	51 (30)	6	8 NS	25	36	97%	87%
Abu Hilal *et al*[54]	35	16	4 (11)	3 (19)	-	-	34	33	75%	67%
Mehta *et al*[72]	30	30	7 (23)	7 (23)	8.4	13.8 NS	38	43	-	-
Baker *et al*[64]	27	85	1 (4)	18 (21)	5.2	9.4[a]	38	40	-	-
Magge *et al*[63]	28	34	28 (100)	34 (100)	11	12 NS	37	45	86%	88%
Kooby *et al*[61]	23	189	23 (100)	189 (100)	13.8	12.5 NS	35	45	74%	73%
Waters *et al*[71]	18	22	2 (11)	11 (50)	11	14 NS	40	60	100%	82%
Limongelli *et al*[56]	16	29	1 (6)	7 (24)	-	-	32	43	94%	93%
Rehman *et al*[76]	8	14	8 (100)	14 (100)	16	14	22	32	88%	86%
Kang *et al*[69]	5	27	5 (100)	27 (100)	8.2	11.3 NS	24	42	100%	85%

[a]$P < 0.05$ *vs* laparoscopic distal pancreatectomy (LDP) group. ODP: Open distal pancreatectomy; NS: Not significant; AC: Adenocarcinoma of the pancreas.

Table 4 Studies comparing laparoscopic distal pancreatectomy and open distal pancreatectomy that include adenocarcinoma of the pancreas but not oncological outcomes *n* (%)

Ref.	Number		Number AC		Tumor size	
	LDP	ODP	LDP	ODP	LDP	ODP
Finan *et al*[65]	44	98	6 (13.6)	23 (22.1)	33	77
Fox *et al*[55]	42	76	2 (5)	2 (3)	29	35
Casadei *et al*[70]	22	22	2 (9)[1]	2 (9)[1]	20	50
Velanovich *et al*[36]	15	41	3 (20)	13 (32)	-	-

[1]Malignant cystic tumors that may represent cystadenocarcinoma. ODP: Open distal pancreatectomy; LDP: Laparoscopic distal pancreatectomy.

in the ODP group[76].

In Tables 3 and 4, studies comparing LDP and ODP and including patients with pancreatic adenocarcinoma of the pancreas are summarized.

DISCUSSION

When feasible, the laparoscopic approach to surgery has spared patients unnecessary suffering. Although initially met with skepticism, the procedure has proven to be useful for treating a great number of benign and malignant tumors[7]. Unfortunately, there is a lack of prospective randomized trials that completely evaluate the outcomes of LDP compared to ODP. There is, however, compelling evidence from retrospective series that LDP reduces bleeding and shortens hospital stay[77-79].

In this review, we have gathered the available studies reporting use of LDP in patients with adenocarcinoma. In this patient group, bleeding, major complications and hospital stay are of importance when compared to ODP. The technique must also provide the same results regarding R0 resections and number of retrieved lymph nodes. In the long term, cancer related survival and recurrence ultimately are important. Unfortunately, the presently available studies are small and non-randomized; in addition, survival data are not always presented. Table 5 shows the available survival data from all studies reporting results from at least 5 patients who underwent LDP for pancreatic adenocarcinoma.

In series that compare LDP and ODP, and in which oncological data is presented, the number of lymph nodes harvested does not vary between the groups[55,57,61-64,67,70,75,76]. Only one of the early studies has deviating results[58]. In reports that provide data on the frequency of negative surgical margins, there is no significant difference between LDP and ODP[48,50,55,57,61-64,67,75,76].

Series that only report LDP have to be compared with the relevant literature presenting results of ODP. The number of lymph nodes harvested in the LDP reports varies between 5 and 19, and most report over 10 lymph nodes harvested. This compares favorably to the results of ODP studies[38,39,53-55,57,68,69]. One LDP series described a strategy of only removing enlarged or suspicious lymph nodes, rather than performing a formal lymphadenectomy. The success of the strategy can be debated, and may explain the low number of lymph nodes harvested in that study[71]. The other report with notably few lymph nodes harvested included data from an early period (2002-2004), and the same group later compared LDP to ODP and reported no significant difference in lymph node harvesting[35,63]. The percentage of negative surgical margins ranges from 88 to 100 in the LDP series, which comparies favorably to ODP[32,38,39,43,53-57,68,69,71]. One of the studies reports a low R0 frequency of 77%, but the same group later reported excellent results in comparison to ODP[35,63].

The current evidence, although somewhat limited, suggests that LDP may be oncologically equal to the gold standard treatment. Notably, the data related to number of lymph nodes resected comes from studies of very few patients and in which the indications for surgery have mainly been benign or low-malignant lesions, rather than the highly malignant adenocarcinoma. This may hold particularly true for the study in which a significant difference was found between LDP and ODP groups,

Table 5 Studies presenting survival data after laparoscopic distal pancreatectomy for pancreatic adenocarcinoma and include at least 5 cases

Ref.	Year	*n*	Survival
Song *et al*[47]	2011	34	2 year 85%
Marangos *et al*[48]	2012	28	3 year 30%
Magge *et al*[63]	2013	28	Median 19 mo
Kooby *et al*[61]	2010	23	Median 16 mo
Fernández-Cruz *et al*[38]	2007	22	Median 14 mo
Rehman *et al*[76]	2013	8	3 year 82%
Giulianotti *et al*[51]	2010	8	Median 15 mo (2 lost in follow up)
Melotti *et al*[39]	2007	7	85% alive after median follow up 26 mo
Kang *et al*[69]	2010	5	Median 28 mo

as the adenocarcinomas were found mainly in the ODP group[58]. In light of this, there may be room for improvement in the oncological outcomes of LDP, provided that resections are performed for suspected pancreatic adenocarcinoma. Additionally, one of the advantages of LDP compared to ODP is a higher rate of splenic conservation, which makes LDP desirable when benign lesions are present. As the splenic hilum contains a number of lymph nodes, LDP reduces the number of lymph nodes resected in those cases. Some of the publications reviewed describe conversions to open surgery, and one of the reasons for converting has been increased suspicion of malignancy. Fortunately, most surgeons no longer believe that converting a laparoscopic operation to an open one is a complication. Instead, it is a sign of sound surgical judgment. The reasons given for conversion to open surgery suggest that malignant features often contribute to the decision[36,38,43,57]. With that in mind, and with the support of the published data, it should be acceptable or even preferable to operate on adenocarcinoma of the body or tail of the pancreas using LDP.

In the studies currently available, there may be a selection bias, for which LPD is selected in patients with smaller tumors. Moreover, the data on long term cancer related survival and recurrence is very limited. On the other hand, some of the publications report that all, or almost all, distal pancreatectomies are performed using LDP, which would eliminate any potential selection bias[69,71]. It would be preferable to conduct a prospective randomized trial on either unselected left sided pancreatic lesions or suspected adenocarcinomas. Until such a study is conducted, the results from series and comparisons using a patient selection ranging from almost exclusively benign or low malignant diseases in the LDP group to unselected patient cohorts must form the basis for these conclusions.

CONCLUSION

LDP likely offers better outcomes than ODP with respect to morbidity and hospital stay. The current data suggests that oncological outcomes of the two procedures are similar and that LDP should therefore be considered a viable option for treating malignancies of the pancreatic body and tail. This conclusion should, however, be confirmed in a prospective, randomized study.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (20): Gastrointestinal surgery

Surgical and interventional management of complications caused by acute pancreatitis

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Abstract

Acute pancreatitis is one of the most common gastrointestinal disorders worldwide. It requires acute hospitalization, with a reported annual incidence of 13 to 45 cases per 100000 persons. In severe cases there is persistent organ failure and a mortality rate of 15% to 30%, whereas mortality of mild pancreatitis is only 0% to 1%. Treatment principles of necrotizing pancreatitis and the role of surgery are still controversial. Despite surgery being effective for infected pancreatic necrosis, it carries the risk of long-term endocrine and exocrine deficiency and a morbidity and mortality rate of between 10% to 40%. Considering high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being explored by gastrointestinal surgeons, radiologists, and gastroenterologists. Since 1999, several other minimally invasive surgical, endoscopic, and radiologic approaches to drain and debride pancreatic necrosis have been described. In patients who do not improve after technically adequate drainage, necrosectomy should be performed. When minimal invasive management is unsuccessful or necrosis has spread to locations not accessible by endoscopy, open abdominal surgery is recommended. Additionally, surgery is recognized as a major determinant of outcomes for acute pancreatitis, and there is general agreement that patients should undergo surgery in the late phase of the disease. It is important to consider multidisciplinary management, considering the clinical situation and the comorbidity of the patient, as well as the surgeons experience.

Key words: Severe acute pancreatitis; Complications; Necrosectomy; Percutaneous drainage; Endoscopy; Laparoscopy

Core tip: The surgery and its timing are contentious regarding treatment of severe acute pancreatitis and related complications. Many studies showed that "early" open surgery has been accompanied often by higher mortality and morbidity rates, and should be the next step in treating severe acute pancreatitis complications, when minimally invasive management fails. In this review article, current treatment options and results are discussed.

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INTRODUCTION

Acute pancreatitis (defined as the acute nonbacterial inflammatory condition of the pancreas) is derived from early activation of digestive enzymes inside acinar cells, with varying compromising of the gland itself, nearby tissues, and other organs. It is well known that several situations develop into acute pancreatitis, but the mechanisms and how those mechanisms develop the disease remain

unclear. Why do some individuals develop edematous pancreatitis and others develop a more severe necrotic pancreatitis? Knowledge regarding pancreatitis pathogenesis may have important implications in prevention and treatment of the disorder. If the early events that generate the inflammatory process are understood - and if pro- and anti-inflammatory factors that modulate the severity of the disease are known - treatment can be implemented so the process will not happen or possible associated complications will be minimized[1].

Acute pancreatitis is one of the most common gastrointestinal disorders requiring acute hospitalization worldwide, with a reported annual incidence of 13 to 45 cases per 100000 persons[2]. In the United States, it is the third most common gastrointestinal disorder requiring acute hospitalization[3]. In the United States alone, acute pancreatitis leads to 270000 hospital admissions annually and in-patient costs exceeding 2.5 billion dollars[4].

It is rare in childhood but may occur at any age (according to recent publications[5,6], median age, 55-58 yr). Acute biliary pancreatitis is more common in women, and alcoholic pancreatitis is more common in middle-aged men[6].

Although most patients with acute pancreatitis recover without sequelae, between 10% to 20% will have a more complicated clinical course with higher risks of morbidity and mortality[7]. Severe acute pancreatitis (SAP) requires prolonged hospitalization, frequently including a stay in the intensive care unit (ICU) because of organ dysfunction[8].

Severe pancreatitis is associated with a mortality of 15% to 30%, whereas mortality from mild pancreatitis is only 0% to 1%, and organ failure is the most important determinant of mortality in acute pancreatitis. However, in approximately 30% of patients with necrotizing pancreatitis, a secondary necrotic infection occurs, mostly 3 to 4 wk after the onset of necrotizing pancreatitis[9]. If left untreated, mortality of infected necrosis approaches 100%[3,10]. Initial treatment of SAP is primarily medical, and these patients require intensive organ support[11,12]. Surgery for SAP is a morbid procedure associated with complications in 34% to 95% of patients, and mortality in 11% to 39%[13,14]. Surgery may lead to long-term pancreatic insufficiency[14,15]. The high mortality rate encountered with surgery reflects the hazards of operating on critically ill septic patients, often with multiorgan failure[16].

Surgery and its timing are the focus of contention when treating SAP. Decades ago, some experts used laparotomy in the early phase of SAP to debride and drain the retroperitoneal infected necrosis[17,18]. However, studies have shown that "early" surgery is often accompanied by higher mortality[19,20], and several studies also have shown that there is success with some SAP patients with retroperitoneal infected necrosis, conservatively managed without high-risk surgical intervention; therefore, many experts advocated delayed surgery[20,21]. In recent decades, higher mortality rates during early surgery resulted from those SAP cases that underwent traditional laparotomy (which may cause severe trauma) to debride and drain the retroperitoneal infected necrosis[22]. After several studies showed that high mortality rates for severe necrotizing pancreatitis came with early surgery, the 2002 International Acute Pancreatitis guidelines recommended avoiding surgical intervention during the first 14 d after onset, unless there was progressive multiple organ failure and clinical deterioration. Subsequent studies have suggested that morbidity and mortality rates can be reduced further if surgery is delayed beyond 28 to 30 d[9], because the extended interval allows sufficient demarcation between normal and necrotic tissue, reducing the risk of inciting overwhelming postoperative septic and systemic inflammatory responses, and the risk of intraoperative injury to surrounding organs and hemorrhage[23].

Faced with high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being increasingly explored by gastrointestinal surgeons, radiologists, and gastroenterologists[24]. As technical ability and endoscopic tools have gradually become more precise, the mortality rates of patients with severe pancreatitis have improved, and there are fewer complications compared to those having open debridement treatment[25]. Percutaneous catheter drainage (PCD), endoscopic transgastric procedures, and a minimally invasive approaches all have been proposed as alternatives to open necrosectomy[16]. When minimal invasive management is unsuccessful or necrosis has spread to locations not accessible by endoscopy, open abdominal surgery is recommended[25].

CLASSIFICATION AND SCORING

The Atlanta Classification system for acute pancreatitis came about as a result of the Atlanta Symposium of 1992, and, despite there being some confusion over definitions, it has been a practical aid for health care providers[11]. Since then, with improvements in the understanding of organ failure and necrotizing pancreatitis, and in diagnostic tools, some revisions have been made through a working group consultation with eleven international pancreatic societies[26]. The fourth draft, in current use, contains a clinical assessment of severity and the previous confusing definitions concerning local complications have been further clarified. The criteria for the diagnosis of acute pancreatitis, the differences between the two forms (*i.e.*, interstitial edematous pancreatitis and necrotizing pancreatitis), the three categories of severity of acute pancreatitis (mildly acute, moderately severe acute, and severe acute)[27,28], and the morphology observed in diagnostic images of pancreatic and peripancreatic collections brought about by complications are now more clearly set out.

Criteria to help in classifying severity are the presence of transient organ failure (that which is present for less than 48 h), persistent organ failure (continuing for more than 48 h), and local (such as, peripancreatic fluid or acute necrotic collections) or systemic complications (such

as exacerbations of underlying comorbidities related to the acute pancreatitis)[29-31].

Scoring systems

Attempts to define objective criteria for assessing disease severity and prognosis were pioneered in the 1970s by Ranson *et al*[32] and Blamey *et al*[33]. The 2 scoring systems include basic laboratory data and clinical variables obtained 48 h after hospital admission. In subsequent years, these scoring systems have found widespread application and have undergone numerous modifications. Several large studies have shown a close correlation between advanced age and nonsurvival in acute pancreatitis[34-36]. Advanced age often is associated with comorbidities (*e.g.*, cardiovascular disease, diabetes, and overall decreased biological resistance)[36], and therefore, increases risk of fatal outcome. Comorbidities have been included in multiple parameter scoring systems such as the Acute Physiology and Chronic Health Examination (APACHE) Ⅱ system, the most widely used index for early risk stratification[37]. Although more recent iterations of this scoring system have been developed, the advantages of the APACHE Ⅱ are its familiarity, its objective nature, and its ability to be calculated at any time during a patient's hospital stay. Use of the APACHE Ⅱ in clinical practice has several important limitations (*e.g.*, the requirement for multiple parameters and an online calculator - versions of which are widely available on the Internet)[38]. As a result, several additional scoring systems have been developed for bedside application.

A more recent scoring system developed for use during the first 24 h of admission to the hospital is the Bedside Index of Severity in Acute Pancreatitis[7]. This system was derived using data from 17992 patients and validated on a population of 18256 patients in the United States. This 5-factor scoring system has a similar accuracy as the APACHE Ⅱ for predicting death in the initial retrospective study and in several subsequent prospective cohort studies[39]. The Bedside Index of Severity in Acute Pancreatitis is a simplified scoring system that can be applied easily in the earliest phases of acute pancreatitis helping identify those patients with an increased risk of death.

DEFINITION AND COMPLICATIONS

Defining the severity of acute pancreatitis

There are three good reasons for defining the severity of acute pancreatitis: the first being diagnosing those patients who may need aggressive early treatment in cases of severe acute form; the second is the identification of patients who may need to be transferred to a specialist care unit; and the third is that placing these patients into sub-groups according to particular complications will aid the specialists to whom they are transferred[26].

Mild acute pancreatitis

Patients without organ failure or complications are classified as having mild acute pancreatitis. They are usually discharged at an early stage, do not need pancreatic imaging, and death as a result of the disease is extremely uncommon[40].

Moderately severe acute pancreatitis

This is diagnosed when transient organ failure, local complications (such as prolonged abdominal pain, leukocytosis, or fever caused by peripancreatic collections, or if the patience can not feed normally), or systemic complications (such as when coronary artery disease or chronic lung disease is made worse as a result) are present. This form of the disease can resolve itself without treatment (when transient organ failure or acute fluid collection is involved) or specialist care may be needed (when extensive sterile necrosis is present, but organ failure is not). The chance of death as a result of this form is lower than in cases of the severe acute form[27].

Severe acute pancreatitis

This is diagnosed when there is persistent single or multiple organ failure, resulting from systemic inflammatory response syndrome caused by cytokine cascades at an early stage[30,31,41,42], which can complicate the pancreatitis, lead to other complications, and increase the risk of death (a 36% to 50% mortality rate), commonly due to infected necrosis, if this is in the first few days of the disease[31,41,42].

One systematic review into deaths caused by necrosis in the absence of organ failure (11% of patients) resulted in a four tier grading of severity being proposed[28], while two large Dutch studies came up with a figure of 6%[43,44]. The differences in morphological characteristics of local complications and their different treatments need to be determined to prevent mortality.

Necrotizing pancreatitis

Necrosis, which affects between 5% and 10% of patients, generally involves both the pancreas and peripancreatic tissue, although sometimes just the peripancreatic tissue, and, even more rarely, only the pancreatic parenchyma.

With patients who have peripancreatic necrosis, as in those with interstitial edematous pancreatitis, the pancreas enhances normally on contrast-enhanced computed tomography, but morbidity is increased and intervention rates are higher[40,45,46]. The progression of both pancreatic and peripancreatic necrosis varies, remaining solid or liquefying, becoming infected or remaining sterile, persisting for a long time or gradually disappearing.

Infected pancreatic necrosis

Both forms of necrosis can become infected, but the majority of evidence shows no certain correlation between its extent, the risk of infection, and its duration, although it is not common in the first week[9,43]. Its diagnosis is crucial as antibiotic and other necessary treatments need to be applied as soon as possible[47]. If computed tomography (CT) scans show up extraluminal gas in the pancreas or peripancreatic tissue, or if biopsies detect

bacteria and/or fungi on Gram stains and cultures, then infection is highly likely[48]. Signs of suppuration may also be evidence of liquefaction and will increase over time. Despite the first version of the Atlanta Classification defining a localized collection of purulent material without significant necrotic material as a pancreatic abscess, the term was found to be unhelpful and is not used in the revised version. Secondary infections have been found to increase the the chances of morbidity and death[49].

Acute pancreatitis complications

Defining organ failure: Organ failure in the respiratory, cardiovascular, and renal systems is defined using the modified Marshall scoring system: a score of two or more for one of these systems is sufficient[50]. This system is preferred over the Sepsis-related Organ Failure Assessment scoring system, used in critical care units, as it is easier to use and gives objective results, although both systems could be used to help stratify the severity of organ failure[51].

Defining local complications: The original Atlanta Classification was useful because it recognized the differences between uncomplicated interstitial pancreatitis and acute pancreatitis with local complications[11]. Local complications (such as acute peripancreatic fluid collection, pancreatic pseudocyst, acute necrotic collection, walled-off necrosis, gastric outlet dysfunction, splenic and portal vein thrombosis, and colonic necrosis) and their clinical consequences are now better understood and described. Signs that these problems may be present are persistent abdominal pain, secondary serum pancreatic enzyme activity increases, organ dysfunction getting worse, and symptoms of sepsis (*i.e.*, fever, white blood cell increases, *etc.*), although imaging may be necessary for correct diagnosis[26].

The definitions of pancreatic fluid collections are based on the revised Atlanta classification by the Acute Pancreatitis Classification Working Group and are described as follows: (1) Acute peripancreatic fluid collections (APFC): these are not connected to necrosis, occur in the first four weeks of acute pancreatitis, are entirely liquid, found in or near the pancreas, and have no fibrous wall or granulation tissue. Those which resolve themselves or have no symptoms need no treatment and are not classed as severe acute pancreatitis; (2) Pseudocyst: a collection of pancreatic juice, containing no solid necrotic material, enclosed by a wall of fibrous or granulation tissue resulting from acute pancreatitis, pancreatic trauma, or chronic pancreatitis. They are a result of the main pancreatic duct or its intrapancreatic branches being disrupted in the absence of pancreatic parenchymal necrosis and causing pancreatic juice to leak persistently and collect, usually after the first month; (3) Infected pseudocyst: this contains purulent liquid with no solid necrotic material (although there may be other solid debris) and can be diagnosed by following the patient's clinical course or through the presence of gas on CT scans; (4) Post-necrotic pancreatic/peripancreatic fluid collections (PNPFC): fluid collections associated with necrotizing pancreatitis, containing fluid and necrotic tissue, which over the course of weeks, evolve into a necrotic fluid collection with liquid and solid debris; and (5) WON: these are formed because of encapsulation of the PNPFC over time in a thickened wall of fibrous or granulation tissue without an epithelial lining at the interface of necrotic tissue, generally maturing after the first month of necrotizing pancreatitis. Walled-off necrosis, resulting from necrotic pancreatic parenchyma and/or necrotic peripancreatic tissue, can be sterile or infected, and there can be many of them, sometimes in locations distant from the pancreas[52]. Walled-off necrosis helps to distinguish the necrotic tissue from the parenchyma, thereby lessening the chances of bleeding and the loss of vital tissue during surgery, although this can result in pancreatic exocrine and endocrine deficiency[53,54].

Bradley *et al*[55] suggested a conservative approach to sterile pancreatic necrosis, although the Acute Pancreatitis Classification Working Group found that patients may continue to be ill even when there was no infection[26,55]. Secondary infection, which usually occurs two to four weeks after primary infection, commonly results in sepsis, multi-organ failure, and patient mortality[56]. High Ranson's and APACHE-Ⅱ scores are good indicators of the possibility of death, and even those with severe sterile necrosis have a high mortality rate if their overall health is not good.

Defining systemic complications

Systemic complications are classed as those arising from already existent complaints, such as coronary artery disease, or chronic lung disease, made worse by the acute pancreatitis. The Acute Pancreatitis Classification Working Group made a distinction between these and persistent organ failure, the latter being the main feature of the severe acute form.

TREATMENT

Management of infected pancreatic necrosis

Pancreatic necrosis surgery, the principles of which were laid out by Moynihan in 1925[57], involves isolating the pancreas from the abdominal cavity and cellular fat spaces, and draining the amassed peripancreatic fluid. The aim is to check the sepsis and control the release of pro-inflammatory mediators. The combination of debriding the necrotic tissue and removing retro-peritoneal debris and exudate is carried out in order to preserve the organ. Four principle surgical methods are recommended: (1) being necrosectomy alongside open packing[58]; (2) being planned, staged relaparotomies with repeated lavage[21]; (3) being closed continuous lavage of the lesser sac and retro-peritoneum[59]; and (4) being closed packing[60].

These days, the third method is most often used to remove post-operative residual pancreatic necrosis as it has the lowest rate of morbidity[24,53]. Surgery has the pos-

sibility of saving the patient's life, but it carries a high risk of morbidity and mortality, between 4% and 10%, and possible long-term endocrine and exocrine deficiency[25]. In addition, timing of surgery has been increasingly recognized as a major determinant of outcome in acute pancreatitis, and there is general agreement that patients must undergo operation in the late phase of the disease. However, the definition of late differs between studies[53,61]. It has been reported that mortality from necrotizing pancreatitis can be reduced by avoiding surgical therapy or by postponing surgery until the late stage of the disease[62].

Despite the availability of several clinical (Ranson criteria, acute physiology and chronic health evaluation Ⅱ score, and APACHE Ⅱ) and radiologic grading systems (Balthazar scoring system, modified computerized tomography severity index), there is no consensus on accurately predicting the best treatment strategy and outcome after acute necrotizing pancreatitis[63-65].

The treatment principles of necrotizing pancreatitis and the role of surgery remain controversial. In the 1990s, more than 60% of patients with the disease were treated surgically[18]. In 1991, Bradley and Allen defined pancreatic necrosis as the principal determinant of survival in acute pancreatitis, but they recommended conservative treatment of sterile necrosis in selected cases[55]. Guidelines of the International Acute Pancreatitis recommend doing a fine-needle aspiration biopsy in patients with necrotizing pancreatitis and signs of sepsis. Once fine-needle aspiration biopsy-proven infection of necrosis has been shown, it is considered an indication for surgery[53].

Recent reports have shown that a subset of patients with SAP developing infected fluid collection, pancreatic necrosis, or pancreatic abscess can be managed by PCD[66]. It was hypothesized that simple drainage with regular-bore (12- to 14-Fr) percutaneous catheters is an effective therapeutic option. This recommendation is based on the premise that is not necessary to remove all necrotic tissue to successfully treat patients with infected pancreatic necrosis. By performing drainage of infected fluid under pressure, the clinical condition might improve and the necrotic tissue may successfully be dealt with by the patient's immune system. The goal of drainage has been to remove infected fluid rather than the necrosis[67]. However, PCD used for infected pancreatic necrosis has been criticized for its poor ability to remove solid debris.

Percutaneous drainage is usually performed under computed tomography, whereas sonographically controlled PCD rarely has been reported[68]. The success rate of percutaneous catheter drainage in infected pancreatic necrosis varies and ranges from 0% to 78%[43,69]. van Baal *et al*[70] reported a meta-analysis, which included 384 patients from 11 studies, of PCD as a primary treatment for necrotizing pancreatitis. Surgical necrosectomy could be avoided in 56% of the patients and the overall mortality rate was 17%. However, infected necrosis was confirmed in only 71% of the patients.

In a recent report, authors aimed to identify factors that led to surgical intervention after initial management with PCD, and also to identify a subgroup of patients where PCD alone would be effective. Twenty-seven patients (38.5%) underwent surgery after initial PCD. Indications for surgical intervention were ongoing sepsis not controlled by interventional radiologic management. In that study, percutaneous catheter drainage achieved sepsis reversal in 62% of patients and complete recovery was achieved without surgical intervention in 48% of patients[16].

Gagner first described minimally invasive surgical treatment of necrotizing pancreatitis in 1996, including laparoscopic retrocolic, retroperitoneoscopic, and transgastric procedures[71]. Over the past 15 years, several other minimally invasive surgical, endoscopic, and radiologic approaches for draining and debriding pancreatic necrosis have been described[23].

A literature search of the MEDLINE database from April 1996 to November 2010 was performed for each of the 4 techniques for minimally invasive necrosectomy: percutaneous therapy (341 studies), endoscopic necrosectomy (574 studies), laparoscopic necrosectomy *via* a transperitoneal approach (148 studies), and retroperitoneal necrosectomy (194 studies). Only cohorts with at least 10 or more patients were included. Twenty-seven studies with 947 patients were examined (8 studies on percutaneous approach; 10 studies on endoscopic necrosectomy; 2 studies on laparoscopic necrosectomy *via* a transperitoneal approach; 5 studies on retroperitoneal necrosectomy; and 2 studies on a combined percutaneous retroperitoneal approach). Finally, the authors advocated a multidisciplinary approach with interventional radiologists, gastroenterologists, intensivists, and hepatobiliary surgeons at tertiary care centers. They concluded that because the comparison data are limited, the minimally invasive approach should be based on location of lesion and individual patient presentation[23].

A prospective, randomized, multicenter trial called the Minimally Invasive Step Up Approach Versus Maximal Necrosectomy in Patients with Acute Necrotizing Pancreatitis (PANTER) was performed in the Netherlands[43]. After diagnosing necrotizing pancreatitis or infected pancreatic necrosis, patients were randomly assigned to either a step-up approach or 2 open necrosectomy. The step-up approach consisted of percutaneous drainage or endoscopic drainage, followed by a minimally invasive retroperitoneal necrosectomy if necessary. A video-assisted retroperitoneal debridement (VARD) with postoperative lavage was performed 3 d after if there was no clinical improvement. Major complications or death occurred in 31 of 45 patients after open necrosectomy (69%) *vs* 17 of 43 patients after the step-up approach (40%). About 35% of patients in the step-up group could be managed with percutaneous drainage only[43].

Similar to the PANTER Trial, there also is a recent, prospective multicenter, single-arm study from the University of Washington. Percutaneous drainage was used

as an initial treatment for infected pancreatic necrosis. If there was a 75% reduction in size based on a follow-up scan 10 d later, the remainder of their treatment would be percutaneous drains alone. If patients did not have a 75% reduction, they were treated with a VARD. Twenty-three percent of patients were treated with percutaneous drains only. Sixty percent of patients were treated with a minimally invasive intervention (*i.e.*, drains with or without a VARD). Mortality at 30 d was 2.5%. The percutaneous approach to infected pancreatic necrosis has been shown to be safe and feasible in multiple retrospective case series. It is noteworthy that 44% of patients reviewed in the studies did not need surgical therapy. What has become increasingly popular is combined percutaneous technique with a VARD as mentioned in the PANTER trial and the Horvath study[72]. These studies not only confirmed a subgroup of patients that can benefit from percutaneous drainage alone but also examined a combined technique in a prospective manner with a relatively larger amount of patients.

Retroperitoneal laparoscopic debridement drainage (RLDD) for treating retroperitoneal infected necrosis in SAP has been rarely reported, and there has been no report regarding comparison of curative efficacy between RLDD and laparotomy. This study showed that RLDD (a minimally invasive procedure) has obvious advantages for treating SAP retroperitoneal infected necrosis. It is safe and effective when done early and can prevent systemic inflammatory response syndrome from progressing further[22].

The overall message of these studies is that in patients who do not improve after adequate drainage, necrosectomy should be performed next. The percutaneous drain, together with the computed tomography scan, can be used as a roadmap for (minimally invasive) necrosectomy. Percutaneous (or transgastric) drainage should be the first intervention, and the indication for drainage should be the same as for surgical necrosectomy[3].

Direct endoscopic necrosectomy (DEN) is a minimally invasive treatment introduced recently for treating infected WON[73]. Using DEN, a stoma is created endoscopically between the enteric lumen and the walled-off fluid collection, allowing insertion of an endoscope into the fluid collection, which allows for an endoscopic necrosectomy. Current data suggest that DEN is a less invasive and less risky alternative to open surgical necrosectomy for managing infected WON and infected pseudocyst with solid debris[74].

Two large, multicenter, retrospective studies demonstrated that necrosis managed using direct transluminal endoscope techniques resulted in a positive prognosis and a high success rate at the beginning[75,76]. Nevertheless, all of the current endoscopic techniques have inherent limitations (*e.g.*, risk of air embolism, endoscopically uncontrollable bleeding, and inadequate drainage through multiple plastic stents) together with early occlusion of the fistulous tract. To overcome these difficulties, Hritz and associates demonstrated a successful method of endoscopic transluminal necrosectomy - a combination of the temporary placement of a self-expanding metal stent into the fistulous tract and daily irrigations of the necrotic cavity with a high-flow water-jet system using a flush knife[77].

Percutaneous techniques, including VARD, need open necrosectomy in a high proportion of patients, and mortality is around 20%[3]. It is now well recognized that most sterile collections do not require intervention (at least in the early phase of disease), and that mortality and morbidity rates after an intervention are time dependent, falling to almost 0% by the stage of a sterile WON. The indication for early intervention for infected necrosis is limited to sepsis control, and there is increasing consensus within this group that some form of minimally invasive approach may enhance outcomes. Conventional management of late pancreatic collections was by open pancreatic cystgastrostomy, but with developments in interventional radiology, therapeutic endoscopy, and minimal access surgery, new techniques have been used as alternatives to this approach[78]. While all have proven to be feasible in small cohort series, there is little evidence to the relative benefits of one method over another for managing APFC[79,80].

Laparoscopic cystgastrostomy (LCG) is used in mature symptomatic collections. It facilitates complete drainage of the collection with a minimal requirement for re-intervention. It also allows simultaneous management of gallstones. Laparoscopic cystgastrostomy should allow a wide debridement of the cyst cavity with the advantages of a minimally invasive approach. Open cystogastrostomy (OCG) is used when an intervention is required on additional intra-abdominal pathology (*e.g.*, enteric stricture or fistula) or where collection anatomy precludes other approaches. Laparoscopic cystgastrostomy allows larger collections to be managed by a one-step intervention, and the solid necrosis to be more effectively drained. Importantly, definitive management of gallstones can be achieved. However, the concept that endoscopic ultrasound (EUS)-guided drainage (the least invasive approach) may be of most benefit in fluid predominant collections requires evaluation within a study format, as experience has shown some APFC with significant necrosis may resolve completely using this approach only. Optimal management of collections with intermediate (size and fluid content) characteristics is not clear, and there may be clinical equipoise regarding whether a laparoscopic or endoscopic cystgastrostomy should be used as the preferred approach. A well-conducted, randomized, controlled study is required to determine which method is most effective in this particular group of patients[78].

In summary, standard treatment for infected pancreatic necrosis is open or laparoscopic surgical drainage. However, on occasions, percutaneous drainage may work well. As recommended by the International Association of Pancreatology Clinical Guideline, drainage should be effectively established when the patient is septic. A step-by-step treatment is proposed by which percutaneous or

endoscopic drainage should be established first and then necrosectomy with drainage through a minimally invasive retroperitoneal access. When this method was compared with open surgery, it offered several advantages including the chance to avoid surgery in some patients, fewer complications, and lower cost[45,53,70].

The alternatives to open surgery should be considered, mainly in frail and critical patients who would not tolerate more aggressive surgery. In clinical practice, it is important to consider the importance of a multidisciplinary management, considering the clinical situation and comorbidity of the patient and the experience of the personnel.

Pancreatic duct breaking: Generally this is produced in the context of pancreatic necrosis because of erosion of the duct. In cases of necrosis, complete or partial pancreatic duct breaking occurs in about 60% of cases. To assess this situation, wirsungography by using computed tomography, nuclear magnetic resonance (spectroscopy), or endoscopic retrograde cholangiopancreatography can be performed. This latter method may be associated with placing a stent, which will favor definitive resolution. Nutritional support and potent antisecretors (*e.g.*, octreotide) should be associated. Collections can be removed by percutaneous or endoscopic drainage. Successful fistula sealing is described using cyanoacrylate or fibrin[81]. If other treatments fail (which is common) surgery is indicated. However, in cases of complete duct rupture, it is rarely successful to access the residual duct in the pancreatic tail. In such cases, a distal pancreatic resection may be curative. Otherwise, internal drainage through a pancreatic-digestive anastomosis, may be necessary[1].

Pancreatic pseudocyst

According to several retrospective studies, the incidence of a pseudocyst after acute pancreatitis varies depending on the definition and methods of detecting a pseudocyst. The incidence ranges from 5% to 16%, and is reported as being higher in patients with underlying chronic pancreatitis[82-84].

Treatment of pancreatic pseudocysts: Fluid collections that appear during disappear spontaneously in 40% to 50% of cases. In about 10% to 15% of cases, these collections persist and become encapsulated, generating pancreatic pseudocysts. A true pancreatic pseudocyst (*i.e.*, without an epithelial lining; the counterpart would be a pancreatic cyst) takes at least 4 to 6 wk from the beginning of symptoms to be encapsulated by a wall formed by inflammatory fibrosis of the adjacent tissues. Few studies have documented the natural evolution of pancreatic pseudocysts. It has been thought that pancreatic pseudocysts more than 6 cm in diameter, or those that persisted for more than 6 wk, should be operated on[1] despite some studies showing that 50% of those which had no symptoms or were smaller than 10 cm resolved of their own accord[84]. It also has been shown that about half of all pancreatic pseudocysts can be solved spontaneously; thus, the attitude has shifted toward a more conservative approach.

Asymptomatic pancreatic pseudocysts may be followed for periods of 6 mo or longer if they do not grow, become symptomatic, or present complications (*e.g.*, hemorrhage, infection, or mechanical compromise of adjacent organs). In these situations, percutaneous, endoscopic, or surgical drainage should be considered. It depends on several factors: patients' general status, size, number, and location of pseudocysts, communication (or not) with the main pancreatic duct, solid necrosis inside (or not), and possible complications[1].

Despite almost 50% of pseudocysts resolving themselves, the remainder can become symptomatic or infected, and may rupture, hemorrhage, develop vascular thrombosis, or obstruct nearby viscera, resulting in the need for some kind of medical intervention[85,86].

A ruptured pseudocyst, if it causes hemorrhaging in the digestive tract, will need immediate treatment, while if it occurs in the peritoneal cavity, it can lead to peritonitis or hemorrhagic shock requiring emergency exploratory surgery[25].

Kim *et al*[84] report spontaneous resolution, including disappearance and a size decrement, was achieved in 71.6% of cases despite the higher proportion of underlying chronic pancreatitis, and there was no significant difference in spontaneous resolution rate between acute and acute-on-chronic pancreatitis groups. Therefore, the wait-and-see policy for more than 4 to 6 wk may be feasible, unless the pseudocysts are associated with symptoms or complications. Although there have been differing results concerning spontaneous resolution of pseudocysts according to the study, size, detection time, and cause of the underlying pancreatic disease were reported as predictive factors[87-89]. The presence of an underlying chronic pancreatitis, an alcoholic cause, and a long interval from symptom onset until admission are risk factors for a pseudocyst, and a single lesion is a predictor of spontaneous resolution[84].

Percutaneous drainage should be avoided in cases of hemorrhage or pancreatic ascites. Surgical treatment (mainly by internal drainage) is reserved for patients in whom percutaneous or endoscopic treatment has failed, those with complications from chronic pancreatitis, those with multiple or giant pseudocysts, or when malignancy cannot be ruled out[90,91].

Hemorrhage or pseudoaneurysm

Hemorrhagic complications: Hemorrhagic complications of acute pancreatitis are fortunately rare; however, they may present in a diversity of forms. Sometimes, upper or lower gastrointestinal bleeding occurs because of gastroduodenitis secondary to adjacent inflammation, bleeding peptic ulcer, pseudocyst rupture into the digestive tract, or drainage of a pseudoaneurysm through the Wirsung duct. In severe cases of acute pancreatitis, bleeding may occur due to intra- or retroperitoneal erosion of

the vessels of the celiac trunk, mainly the splenic artery. Diagnosis may be established by angiography or angio-computed tomography. Angiography, besides identifying the bleeding point, sometimes allows embolization that may stop bleeding. If this method fails, definitive treatment must be surgery[92].

Ischemic complications (either local or related to remote vascular events) and venous or arterial complications - specifically splanchnic thrombosis and associated varices - are a major cause of morbidity and mortality[93]. The reported frequency of pulmonary embolism in acute pancreatitis is rare. The thrombohemorrhagic complications in pancreatitis play a tremendous part in developing its most severe forms and fatal outcomes. Early recognition and investigation of thromboembolism is imperative because accurate diagnosis and timely radiologic interventional procedures reduce mortality. Early treatment with intravenous heparin or thrombolysis is effective. Vascular filter insertion may be a life-saving measure for such patients[94].

Pseudoaneurysm

Pseudoaneurysm is a rare but potentially fatal complication of acute pancreatitis. The risk of rupture is as high as 37%[95]. The arteries involved include, in order of frequency, the splenic (40%), gastroduodenal (20%), pancreaticoduodenal (20%), gastric (5%), and hepatic (2%)[96]. The pathogenetic mechanism is secondary to degradation of the vessel wall by pancreatic enzymes released from a destroyed pancreatic duct, resulting in a primary formation of a pseudoaneurysm or rupture of the vessel into a pre-existing pseudocyst, which then converts into a pseudoaneurysm. Pseudoaneurysms present symptoms such as gastrointestinal bleeding (60%), abdominal pain (50%), and splenomegaly or pulsatile abdominal tumors (5%), and spontaneous regression also have been reported[97,98].

Generally developing intracystically, they are usually diagnosed *via* angiography, which is used for locating and treating with embolization (with a high technical success rate of 93%-100%, and low 24 h and 30 d re-bleeding rates - 4% and 17%, respectively, Kalva *et al*[99]), but it should also be borne in mind when a pancreatitis patient is undergoing a CT scan. Gonzalez *et al*[100] have also demonstrated that lipiodol with n-butyl cyano-acylate injected using endo-ultrasonography can be successful. If these techniques are not successful or if re-bleeding occurs, then surgery is required[25].

Necrotizing pancreatitis and pseudocysts involving the pancreatic tail appear to predispose patients to splenic complications[101]. The incidence of pseudocyst extension into the spleen has been estimated at around 1%. Erosion of noncystic pancreatic inflammation occurs less commonly[102,103]. In a series of 500 patients with chronic pancreatitis, splenic complications were found in 11 patients (2.2%), four of whom presented with splenic rupture. Five patients had intrasplenic pseudocysts and 2 had intrasplenic subcapsular hematomas[104]. A series of 159 CT scans performed on 100 consecutive patients with acute pancreatitis found splenic infarcts in 10 patients and subcapsular hemorrhage in 2 patients[105]. Another series of 238 patients with pancreatic pseudocysts found 14 patients (5.9%) with splenic parenchymal involvement[106].

Management of patients with subcapsular hematomas and/or splenic parenchymal pseudocysts is by conservative approach, percutaneous drainage, or surgery[106]. The hemodynamically unstable patient with splenic rupture or hemoperitoneum requires emergency laparotomy and either splenectomy or distal pancreatosplenectomy, which can reduce the risk of pancreatic leak or fistula formation[104,106]. In hemodynamically stable patients, the decision for intervention should be based on clinical parameters rather than computed tomography imaging alone. A clinically stable patient with improving symptoms and resolving clinical signs can be managed conservatively with the intent of splenic conservation. Follow-up is by serial ultrasound or computed tomography scans, which can show spontaneous regression. Time for resolution varies from 1 wk to 4 mo depending on the severity of the underlying pancreatitis[107].

Chylous ascites

Pancreatitis is a rare cause of chylous ascites formation. It is believed that either lymph may actually leak through destroyed lymphatics because of pancreatic enzyme erosion or that chylous accumulation results from exudation of chyle, caused by the obstruction of lymphatic channel flow secondary to severe inflammatory changes that take place in the retroperitoneal space surrounding the pancreas[108]. Most cases involve chronic pancreatitis, though acute pancreatitis also has been recognized as the causative reason, with the first such report dating to 1984[109]. Since that time, only a few cases of chylous ascites secondary to acute pancreatitis have been documented. In almost all, the presence of chyle into the peritoneal cavity was discovered some time after the episode of pancreatitis, usually days or weeks[108]. However, Khan *et al*[110] reported a case of acute hyperlipidemic pancreatitis (with normal serum amylase) that presented with acute chylous peritonitis and was treated conservatively. Smith *et al*[111] operated on a patient with relapsing pancreatitis and acute chylous ascites formation caused by a clinical resemblance with appendicitis.

Therapeutic choices may vary in accordance with the underlying pathology.

Thorough lavage of the abdomen and adequate drainage is an excellent treatment modality for acute chylous peritonitis, because resolution of chylous ascites usually occurs within the next few days. However, successful conservative treatment also has been reported[112]. Conservative treatment requires proper preoperative diagnosis, which is often difficult because of the exceptional rarity of this condition and its resemblance to other surgical urgencies that call for immediate laparotomy. Long-term fasting, supported by total parenteral nutrition, frequently offers resolution. Alternatively, a high-protein low-fat diet

is effective at reducing the amount of chyle produced. Administration of octreotide is controversial[108].

In summary, the mortality rate for severe acute pancreatitis stands at between 15% and 30%, while if the between 5% and 10% of patients with parenchyma or peripancreatic necrosis are left untreated and it becomes infected, the mortality rate can be as high as 100%. The surgical methods and its timing are contentions regarding treatment of severe acute pancreatitis. Many studies showed that early surgery often was accompanied by higher mortality and morbidity rates. Faced with high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being explored by gastrointestinal surgeons, radiologists, and gastroenterologists. In cases where there are severe acute pancreatitis complications, minimally invasive treatment is unsuccessful, or if there is widespread necrosis in locations not easily reached using other techniques, then traditional open surgery is strongly recommended.

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REVIEW

Development of minimally invasive techniques for management of medically-complicated obesity

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Abstract

The field of bariatric surgery has been rapidly growing and evolving over the past several decades. During the period that obesity has become a worldwide epidemic, new interventions have been developed to combat this complex disorder. The development of new laparoscopic and minimally invasive treatments for medically-complicated obesity has made it essential that gastrointestinal physicians obtain a thorough understanding of past developments and possible future directions in bariatrics. New laparoscopic advancements provide patients and practitioners with a variety of options that have an improved safety profile and better efficacy without open, invasive surgery. The mechanisms of weight loss after bariatric surgery are complex and may in part be related to altered release of regulatory peptide hormones from the gut. Endoscopic techniques designed to mimic the effects of bariatric surgery and endolumenal interventions performed entirely through the gastrointestinal tract offer potential advantages. Several of these new techniques have demonstrated promising, preliminary results. We outline herein historical and current trends in the development of bariatric surgery and its transition to safer and more minimally invasive procedures designed to induce weight loss.

Key words: Obesity; Bariatrics; Bariatric surgery; Weight loss; Endoscopy

Core tip: Obesity and its associated co-morbidities are on the rise worldwide and have reached epidemic proportions. Surgical procedures have been developed and refined to manage obesity. Bariatric surgery is now the preferred modality of therapy for medically-complicated obesity. Attempts to replace invasive bariatric techniques are the driving factors behind studies of newer, minimally invasive procedures. Our therapeutic armamentarium continues to expand for treatment of morbid obesity and its medical complication as new research is completed and novel minimally invasive techniques are assessed. Preliminary results in several of these areas are promising and provide practitioners with a potential future array of options and modes of therapy.

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INTRODUCTION

Over the past several decades, obesity has increasingly

become a major health care concern. From 1980 to 2008, the global trend has shown a rapid increase in the mean body mass index (BMI) by a rate of 0.4 kg/m^2 per decade[1]. The highest mean BMI among high-income countries is found in the United States[1]. According to the Centers for Disease Control and Prevention National Center for Health Statistics, 35.7% of adults in the US were obese in 2009 and 2010[2]. Although the overall trend continues to rise, the prevalence of obesity may be reaching a plateau as it did not significantly change in 2009 and 2010 when compared with 2003 to 2008[3]. While the increasing prevalence of obesity may be slowing, morbid obesity continues to rise at a brisk rate in the United States[4].

The rise in obesity is a major contributing factor to the growing prevalence of type 2 diabetes mellitus[5,6]. The risk of developing type 2 diabetes mellitus increases proportionally with increasing BMI as each unit of increased BMI correlates with an approximately 12% increase in risk[7]. Obesity has also been significantly associated with high blood pressure, high cholesterol, asthma, arthritis, and overall poor health status[8]. Both the United States Preventive Services Task Force and the National Institutes of Health (NIH) recommend treatment of obesity through improvement of diet or nutrition, increasing physical activity, and behavioral interventions[9,10]. However, treatment of morbid obesity with bariatric surgical procedures has been shown to be significantly superior to intensive medical programs and has demonstrated improvement in type 2 diabetes mellitus indices, sustained weight loss, improvement in quality of life, and amelioration of several cardiovascular risk factors[11-15].

Guidelines set forth by the NIH recommend bariatric surgery as a treatment option for obesity in patients with a BMI $\geqslant$ 40 kg/m^2 or for patients with BMI $\geqslant$ 35 kg/m^2 and co-morbidities, for whom other therapies have failed[9]. Some experts have advocated for less stringent guidelines for bariatric surgery as a treatment modality. Although there is data to suggest patients with BMI $\leqslant$ 35 kg/m^2 would benefit from bariatric surgery[16,17] more extensive and robust data is needed before such widespread recommendations can be made[18].

Bariatric surgical options are numerous and continue to expand with advancing technology due to the availability of less invasive procedures. In patients who undergo bariatric surgery, the utilization of minimally invasive, laparoscopic surgery provides multiple advantages over the older, open surgical methods. Laparoscopic bariatric surgery provides earlier ambulation after surgery, less postoperative abdominal pain, lower postoperative risks of pneumonia and deep venous thrombosis, decreased length of hospital stay, improved cosmetic appearance, lower risk of postoperative wound complications (including those of infection and hernia development), and an earlier return to societal activities, including their job activities.

In addition to the increasing number of surgical options, endoscopic techniques that have had varying degrees of success in the treatment of morbid obesity are becoming more widely studied. An array of approaches has been explored ranging from duodenal and intralumenal intestinal electrical stimulation to natural orifice translumenal endoscopic surgery (NOTES). In this article, we review the hypothesized pathophysiology, surgical procedures, and endoscopic procedures currently available as well as future directions in the field of bariatrics.

TYPES OF BARIATRIC SURGERY AND THEIR PROPOSED EFFECTS

The first surgical procedure for the purpose of inducing weight loss was described by Dr. Viktor Henrikson in a 1952 case report discussing resection of the small intestine[19]. Bariatric surgery for the treatment of obesity was studied by Kremen *et al*[20] in 1954 using dogs as an animal model. They subsequently performed a jejunoileal bypass on a human subject. The early forms of bariatric surgical procedures had high rates of morbidity and mortality. With evolution of the field, bariatric surgeries have been altered to increase safety and to allow for fewer adverse effects. The annual number of bariatric surgical procedures performed worldwide has surged since 1998[21], and has now become a widely available and accepted modality for the treatment of morbid obesity. Approximately 146000 bariatric surgeries were performed worldwide in 2003[22]. The upward trend has continued with greater than 340000 annual bariatric surgeries worldwide in 2008 and 2011[23].

The most common bariatric surgical procedures are outlined in Figure 1. Roux-en-Y gastric bypass (RYGB) is the most commonly performed bariatric surgery worldwide, including in the United States/Canada, Europe, and Latin/South America. Adjustable gastric banding (AGB) is currently the third most common bariatric surgical procedure worldwide, and by 2011 AGB was less commonly utilized (17.8% internationally) compared to 2003 (24.4% internationally)[23]. The major reason for the decline in utilization of AGB has been the pronounced upward trend in the percentage of vertical sleeve gastrectomy (SG) surgeries performed between 2003 (0% internationally) and 2011 (27.89% internationally)[23]. This shift in the choice of laparoscopic surgical procedures may be due to a more rapid and more substantial weight loss after SG; it may be due to the removal of the risk related to the chronic implantation of an adjustable band; it may be due to the removal of requirement of follow up adjustments in individuals with adjustable bands; it may be related to an absence of urgent return visits following the overfilling of adjustable bands.

The initial versions of AGB were introduced in the 1980s and then further refined to insertion with a laparoscopic technique in 1993. The advantage of AGB is that it does not involve reduction or stapling of any portion of the stomach, and it does not involve alteration of the anatomy with the creation of a bypass of segments of the small intestine. It is a relatively safe procedure with low morbidity and mortality, and offers patients the potential for minimally invasive adjustments of the band or

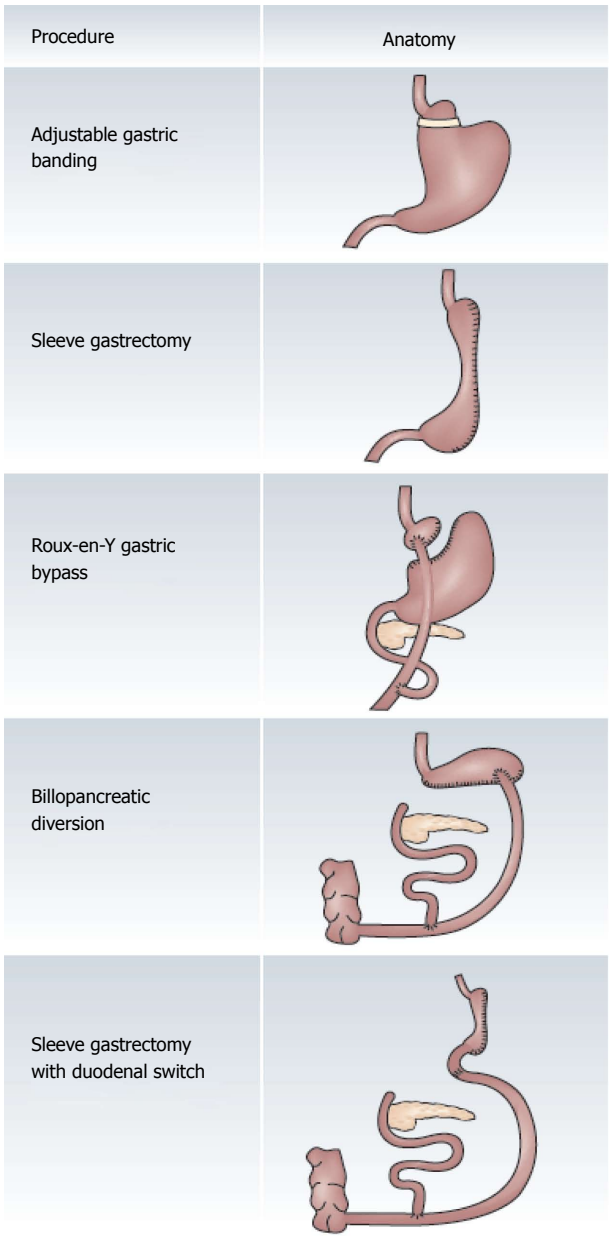

Figure 1 Comparison of bariatric surgical procedures. The major restrictive bariatric procedures depicted at the top of the figure include the adjustable gastric banding and sleeve gastrectomy. The restrictive and malabsorptive procedures are depicted in the bottom of the figure. There are elements of both food restriction as well as malabsorption in patients who have undergone either Roux-en-Y gastric bypass or sleeve gastrectomy with duodenal switch. The major malabsorptive bariatric procedure is the biliopancreatic diversion. Reprinted by permission from Ref [34].

even a reversal of the procedure. Although the efficacy is not as dramatic as other bariatric surgical procedures, the majority of patients experience greater than 40% of excess weight loss (EWL) within one year[24,25]. The mechanisms associated with weight loss in AGB are not well understood. Weight loss was initially thought to be related to a restrictive mechanism; however, several studies indicated that appetite reduction plays an important role[26,27]. The reasons for this suppression of appetite remain unclear.

SG is gaining popularity worldwide and is becoming one of the most commonly performed bariatric surgical procedures. The gastric fundus and body are surgically excised, leaving a narrow, tubular stomach along the lesser curvature of the stomach. It allows for an immediate restriction of caloric intake without placement of a foreign body, or the need for adjustments. Although SG is irreversible and has a relatively increased operative risk compared with other bariatric surgeries, the procedure is often less time consuming and requires less time under general anesthesia. The long-term data on the efficacy and durability of this procedure is lacking. Patients experience 65% of EWL within one year and maintain approximately 50% EWL within 6 years[28-30]. Similarly to AGB, weight loss after SG was first theorized to be due to a restrictive mechanism; however, recent studies have indicated that hormonal changes play a critical role[31-33].

RYGB continues to be the most commonly performed bariatric surgery performed worldwide and comprises nearly 50% of all procedures performed. Despite its popularity, there is a recent downward trend in the number of RYGB procedures being performed annually worldwide[23], possibly due to the increasing favor gained by SG. In this procedure, a small gastric pouch is created and directly connected to the jejunum, thus bypassing a large portion of the stomach and duodenum. Three channels are formed: the continuous digestive tract (termed the Roux limb), the biliopancreatic limb, and the common channel (between the jejunojejunostomy and the ileocecal valve). Patients with a short common channel (< 120 cm from the ileocecal valve) are at greater risk of developing severe malabsorption than those with a longer common channel[34]. RYGB effectively reduces the gastric reservoir to provide a restrictive component for weight loss, and also creates a malabsorptive component by excluding a variable portion of the duodenum and jejunum to inhibit fat absorption[35]. Hormonal effects may also provide a significant contribution to the efficacy of RYGB related weight loss[36]. In an examination of the potential benefit of probiotics, Woodard *et al*[37] randomized 44 individuals after Roux-en-Y gastric bypass to receive either placebo control or 2.4 billion *Lactobacillus* species daily. At 6 mo, there were no differences in weight loss between these two groups. There was reduced breath hydrogen levels in subjects who received daily Lactobacillus, supporting suppression of small intestinal bacterial overgrowth. The importance of gastric bypass surgery in improving patients' quality of life was supported by improvements in gastrointestinal quality of life surveys in both of the groups. Scopinaro *et al*[38] initially developed biliopancreatic diversion (BPD) in the mid-1970s. This procedure involves a distal gastrectomy with closure of the duodenal stump. The jejunum is divided and the distal limb (termed the roux limb) is anastomosed to the proximal stomach, while the proximal limb (termed the biliopancreatic limb) is anastomosed to the ileum. Initial versions of this procedure had high morbidity with patients often experiencing dumping syndrome. To resolve this problem, a variation of the procedure, termed biliopancreatic diversion with duodenal switch (BPD/DS),

was developed to preserve the pylorus and control gastric emptying. To help maintain the restrictive component of BPD/DS, a partial gastrectomy of 70%-80% of the greater curve of the stomach is performed in a gastric sleeve configuration[39].

Vertical banded gastroplasty (VBG) is a procedure that was developed in the 1970s and 1980s by surgeons who were attempting to develop a bariatric surgical procedure without significant morbidity. Designed to be a purely restrictive procedure, the gastric anatomy is altered to restrict the daily caloric intake and cause early satiety. The fundus of the stomach is separated with a stapled partition, creating a vertical pouch along the proximal lesser curvature. A narrow band of polypropylene mesh is used to reinforce the outlet of the pouch. This procedure is not technically challenging for most surgeons and has less morbidity than gastric bypass. Despite the excellent short-term outcomes with EWL of 60% or more at one year, patients often do not maintain this level of weight loss and have only 40% of EWL ten years after their surgery. The failure to maintain long-term weight loss is often attributed to dehiscence of the staple line, allowing food to re-enter the main body of the stomach and thus negating the effects of surgical restriction[19]. Because of the aforementioned reasons, this procedure has largely fallen out of favor and accounted for only 0.7% of bariatric procedures worldwide in 2011[23].

GASTROINTESTINAL PHYSIOLOGICAL BASIS FOR WEIGHT LOSS PROCEDURES

Weight loss observed in patients with malabsorptive and/or restrictive bariatric procedures was initially believed to be the result of readily apparent mechanisms. The procedures that have a restrictive component (AGB, VBG, SG, and RYGB) were designed to drastically decrease the size of the stomach. According to this theory, patients experience early satiety with smaller meal portions due to the reduced gastric capacity. If restriction of the gastric capacity was the only mechanism involved in weight loss after bariatric surgery, the body would likely compensate by increasing the intake of calorie-dense food and meal frequency. Paradoxically, after RYGB and vertical gastroplasty, patients were noted to have lower calorie food preferences and consumed significantly smaller meal portions[40,41]. Individuals with VBG do not experience the same degree of effects as they tend to consume a slightly higher proportion of dietary fat and calorie-dense foods when compared to patients after RYGB[42]. There is an abundance of data showing that RYGB causes more significant amounts of weight loss and is more effective at reducing appetite than VBG[13,43-46]. These findings are suggestive of an alternative, underlying mechanism which contributes to weight loss, rather than merely being induced by simple restriction of the gastric reservoir.

Evidence has been mounting in support of these less well defined physiological changes that, in addition to causing weight loss, also help improve co-morbidities such as type 2 diabetes mellitus. In a large meta-analysis including 22094 patients after bariatric surgery, Buchwald *et al*[43] found that 1417 of 1846 patients had complete resolution of type 2 diabetes mellitus. Further categorization by type of bariatric surgery shows drastic differences in efficacy. Of those with AGB and VBG, 47.8% and 68.2% had resolution of type 2 diabetes mellitus respectively, contrasted with 83.8% of patients with RYGB, and 97.9% with BPD or BPD/DS. A plausible theory to explain this observation is that RYGB, BPD, and BPD/DS affect glucose homeostasis by a weight loss-independent mechanism.

Time course studies have further supported the likelihood of physiological and hormonal changes contributing to the efficacy of bariatric surgery. Wickremesekera *et al*[47] assessed 71 obese patients undergoing RYGB who were categorized into three groups: diabetics, impaired glucose tolerance, and normal glucose tolerance. All three groups had insulin resistance prior to surgery (diabetic patients had greatest insulin resistance), and they all showed improvement of insulin resistance within six days of RYGB. Of the 31 diabetic patients included in the study, only three individuals required medications for treatment of diabetes mellitus at the time of their discharge. Given the lack of any appreciable weight loss six days after RYGB, this finding is consistent with a humoral mechanism for control of hyperglycemia. Similarly, Tsoli *et al*[48] studied 12 patients undergoing BPD. All of the patients discontinued their anti-diabetic medications postoperatively, and at one month, only one patient still had diabetes. To aid our consideration of the potential mechanisms to explain these clinical findings, the proposed physiological roles of regulatory peptide hormones in the gastrointestinal tract are summarized in Table 1.

Two major hypotheses have been proposed to explain the immediate beneficial effects on type 2 diabetes mellitus seen in patients after duodeno-jejunal bypass (as performed with RYGB, BPD, and BPD/DS) in humans. The "foregut hypothesis" suggests that bypass of the proximal intestines exerts an anti-diabetic effect by disallowing calories from being exposed to the foregut, and thus, preventing release of a diabetogenic, hormonal mediator. An alternative hypothesis is termed the "hindgut hypothesis." This theory proposes that an increased delivery of calories and nutrients to the distal jejunum and ileum increases the secretion of enteroendocrine hormones, including peptide YY and glucagon-like peptide-1 (GLP-1)[36]. Although the proper mechanism(s) remain unknown, an animal model created to study the differences between these theories supports the foregut hypothesis. This study showed that duodeno-jejunal bypass directly ameliorates type 2 diabetes mellitus independently of food intake, weight, malabsorption, or nutrient delivery to the hindgut. Therefore, unknown or undiscovered factors from the proximal portions of the bowel may contribute to the pathophysiology of type 2 diabetes mellitus[49]. The proposed effects of bariatric surgery on peptide hormones in the gastrointestinal tract are summarized in Table 2.

Table 1 Proposed physiological roles of gastrointestinal peptide hormones

Hormone	Localization	Proposed physiological roles
Peptide YY	Ileum and colon	Inhibits gastric emptying/ intestinal transit; possible blockade of hunger
Glucagon-like Peptide 1	Ileum and colon	Delays gastric emptying; Incretin: Augments insulin secretion
Glucose-dependent insulinotropic peptide	Duodenum and proximal jejunum	Incretin: Augments insulin secretion
Oxyntomodulin	Ileum and colon	Blockade of hunger; inhibits gastric secretion and emptying
Ghrelin	Gastric fundus	Appetite stimulant; increases gastric motility
Pancreatic polypeptide	Pancreas	Suppresses food intake; inhibits gastric emptying
Cholecystokinin	Duodenum and proximal jejunum	Suppresses food intake; initiates satiety; inhibits gastric emptying

Table 2 Gastrointestinal peptides hormones after bariatric surgery

Hormone	Bariatric surgery	Blood hormone levels
Peptide YY	RYGB	Increased postprandially
Glucagon-like peptide 1	RYGB	Increased postprandially
Glucose-dependent insulinotropic peptide	RYGB and AGB	Increased postprandially
Oxyntomodulin	RYGB	Increased postprandially
Ghrelin	RYGB and SG	Low Fasting and Postprandially
Pancreatic polypeptide	RYGB	Decreased
Cholecystokinin	VBG	Increased

RYGB: Roux Y gastric bypass surgery; AGB: Adjustable gastric band; SG: Vertical sleeve gastrectomy; VBG: Vertical banded gastroplasty.

Peptide YY

The hypothalamus regulates food intake and satiety with neuropeptides. Peptide YY is a member of the neuropeptide Y family of biologically active peptides and is an important hormone in gut-brain communication[50]. Peptide YY is almost exclusively expressed in the gastrointestinal tract and is primarily secreted from mucosal enteroendocrine L-cells[51]. As shown by our group, Peptide YY is mainly concentrated in the ileum and colon, with the highest concentrations found in the distal colon[52]. Exercise and food intake stimulate the release of Peptide YY causing levels to peak within two hours and remain elevated for several hours[53]. Peptide YY interacts with Y receptors and has an inhibitory effect on gastric emptying and intestinal transit. Expression of Peptide YY has been demonstrated in the hypothalamus and pituitary tissues of human brains, which may suggest a role for Peptide YY as a neurotransmitter involved in the regulation of appetite and energy expenditure[54].

Lower fasting plasma Peptide YY concentrations have been exhibited in obese individuals when compared to their lean counterparts[55,56]. After VBG, patients have continually increasing levels of basal Peptide YY concentrations at 6 and 12 mo that correlate with their weight loss and approach the control levels. However, these patients do not have significant postprandial spikes one hour after meals as would be expected with a normal physiological response. This implies that the cause of increases in the basal Peptide YY concentrations may be related to their weight loss rather than the surgical procedure. Although basal Peptide YY concentrations are highest in lean controls, there is no statistical difference compared to patients with RYGB. In response to a meal, RYGB patients have an exaggerated spike in Peptide YY levels. Postprandial Peptide YY concentrations start to rise almost immediately and have been demonstrated to increase as early as two days after RYGB[57] and one week after BPD[58]. At 90 min after a meal, they have approximately two to three fold higher concentrations compared with lean and matched controls[59].

GLP-1

Incretins are gastrointestinal hormones that augment insulin secretion from the beta cells of the islets of Langerhan in the pancreas, even prior to elevations in blood glucose. There are currently two hormones that are classified as incretins: glucagon-like peptide or GLP-1 and glucose-dependent insulinotropic polypeptide (GIP). Similar to Peptide YY, GLP-1 is produced by enteroendocrine L-cells primarily located in the distal ileum and colon[60]. There is biphasic secretion of GLP-1 from the gut in response to food intake. The early phase occurs within 10-15 min after ingestion of food and the late phase peaks at approximately 30-60 min[60]. GLP-1 receptors have been identified in a wide range of tissues including the lung, heart, kidney, stomach, intestine, pancreatic islets, and the peripheral and central nervous systems[61]. Similar to Peptide YY, GLP-1 has been implicated in the regulation of food intake and energy homeostasis. GLP-1 delays gastric emptying and acts as a physiological regulator of food intake and appetite, increases satiety, reduces hunger, and plays a role in glucose homeostasis[62-65]. Although there are GLP-1 receptors in the intestines and in numerous areas of the brain, the exact roles of this peptide have not yet been delineated. Several studies have supported the theory that GLP-1 exerts its effects on delaying gastric emptying through the vagus nerve[66-68]. However, animal studies have supported the notion that the effects of GLP-1 may not be solely dependent on vagal afferent signaling, but rather may also involve receptors within the central nervous system[60].

Although AGB also induces satiety with optimal adjustment[26], it has not been shown to be associated with the exaggerated postprandial hormonal responses seen after RYGB[69]. As seen with Peptide YY, patients have an increased and exaggerated postprandial GLP-1 response after RYGB when compared to lean and obese controls. These hormonal effects, however, were not observed in patients that have lost an equivalent amount of weight

from AGB[70]. Elevations of both GLP-1 and peptide YY have been associated with an attenuated appetite after RYGB. Subsequent inhibition of the release of these hormones with octreotide (a somatostatin analogue) causes increases in food intake in patients after RYGB[57]. This finding suggests that these gut hormones play a crucial role in the reduced food intake after RYGB, but not after AGB. The enhanced hormone level response is sustained two years after surgery and may help to explain the maintained weight loss seen with RYGB[71].

GIP

GIP is synthesized and secreted from intestinal K-cells, a majority of which are located in the duodenum and proximal jejunum[72]. It is secreted in response to ingestion of nutrients, especially glucose and fat, and is significantly higher after ingestion of large meals compared to small meals[73]. The rate of GIP release is related directly to nutrient absorption, rather than the mere presence of intraluminal food and nutrients. Therefore, malabsorption and decreased nutrient absorption reduces the secretion of GIP[61]. In the pancreas, GIP inhibits β-cell apoptosis and stimulates proliferation of β-cells and glucose-dependent insulin secretion[74]. Although one of the main functions of GIP is its effect on the pancreas, GIP receptors have been identified in the stomach, small intestine, heart, lung and brain[72].

Patients with type 2 diabetes mellitus have a severely reduced incretin effect, which is likely multi-factorial in origin. Despite normal or even increased basal and postprandial GIP concentrations[75], its insulinotropic effect is greatly diminished[76,77]. GIP has a sharp postprandial trajectory after RYGB compared to matched controls with caloric restriction. The increment in GIP from baseline to its peak levels were larger and occur much more swiftly in patients after RYGB than with caloric restriction[78]. Despite the sharp increases seen after RYGB, comparisons with AGB reveal even higher concentrations of postprandial GIP after AGB than RYGB[69]. One study compared patients with AGB and SG to patients with RYGB and BPD/DS (defined in that study as restrictive procedures *vs* malabsorptive procedures, respectively). Although patients with malabsorptive procedures experienced increased concentrations of GIP, it was not statistically significant. Those with restrictive procedures had much larger longitudinal increases at one month and three months after surgery[79]. These findings may be due to bypass of the K cells in the duodenum and proximal jejunum with procedures such as RYGB and BPD/DS[79].

Oxyntomodulin

Oxyntomodulin is another product of the L-cells found in the distal portions of the gastrointestinal tract and is secreted in response to food ingestion[80]. It binds to the same receptors as GLP-1, but with lower affinity. Oxyntomodulin is an anorectic hormone. After a meal, its circulation increases in proportion to the caloric intake and peaks within 30 min, remaining elevated for several hours[53]. It can inhibit gastric acid production and pancreatic secretion, and it delays gastric emptying[81]. Administration of oxyntomodulin causes reductions in food intake in lean and obese individuals and promotes a reduction in body weight[82,83]. The changes in oxyntomodulin concentrations correlate with the changes seen in GLP-1 and peptide YY, which makes it difficult to distinguish its effects from those of other peptides. Various studies have supported the possibility that these three hormones work in synergy and act as a powerful hormonal triad contributing to post-surgical weight loss[80]. After RYGB, the reported results are similar to that reported with GLP-1 and Peptide YY. Patients experience an exaggerated rise in oxyntomodulin levels, more than two-fold higher than controls, in response to an oral glucose load[84].

Ghrelin

Ghrelin is an orexigenic (appetite stimulant) hormone that is secreted from the X/A-like cells within the gastric oxyntic cells[85]. It appears to increase gastric motility, stimulates the hypothalamo-pituitary-adrenal axis, and enhances cardiac contractility[86]. Ghrelin is principally secreted from X/A-like cells within the fundus of the stomach, but may be found in smaller amounts in the duodenum, jejunum, and ileum[80]. Ghrelin also functions as a neurotransmitter and is expressed within the arcuate nucleus of the hypothalamus and periventricular regions[87].

In humans, ghrelin demonstrates a diurnal rhythm by rising throughout the day to a zenith at 0100, but then falling overnight to a nadir at 0900. Secretion of ghrelin is stimulated by fasting, by cholecystokinin (CCK), and by gastrin, and is inhibited by food ingestion, by somatostatin, and by growth hormone[80]. There is a clear rise in preprandial ghrelin concentrations followed by a postprandial decline[88]. Patients with anorexia nervosa have high fasting plasma ghrelin concentrations with normalization of levels after weight gain[89]. Similarly, diet-induced weight loss also leads to increases in plasma ghrelin concentrations[90]. Although ghrelin levels decrease postprandially in normal-weight subjects, obese individuals demonstrate a much smaller drop in ghrelin levels after meals[91,92]. Circulating ghrelin concentrations have been found to be negatively correlated with BMI[93]. These findings suggested that ghrelin may be a strong contributor in the regulation of and pathophysiology of obesity.

The role of ghrelin post-bariatric surgery is complex and difficult to assess. The findings are dependent on several variables including the different types of bariatric surgeries, various levels of weight loss, and different intervals after surgery. One of the first reports of decreased ghrelin concentrations after RYGB was by Cummings *et al*[90] and similar results have since been replicated by several other studies[94-96]. They compared patients after RYGB to lean volunteers and matched obese patients that lost weight through diet. RYGB resulted in lower fasting, postprandial, and interprandial plasma ghrelin

levels compared with obese and lean patients. The decline in ghrelin is almost immediate after RYGB with levels demonstrated to be significantly lower as early as one day after surgery. This decline is maintained for at least two years[97].

Similarly, ghrelin plasma levels are noted to decrease immediately with SG as early as one day after surgery, but not with AGB. The ghrelin levels remain low for at least six months after SG[98], but in patients with AGB, levels may actually increase after the surgery[26,98,99]. Plasma concentrations of ghrelin are also increased after BPD[100]. However, when BPD/DS is performed, which includes resection of the gastric fundus, a decrease in ghrelin concentration is observed[101]. A series of studies have demonstrated that bariatric procedures preserving the gastric fundus do not result in decreases in plasma ghrelin, whereas procedures that resect the gastric fundus lead to lower fasting plasma ghrelin concentrations[59,96,102-104]. The exact role and overall effect of ghrelin on weight loss and obesity remains unclear. Despite findings that procedures causing decreases in levels of ghrelin (RYGB and SG) result in better weight loss, effective weight loss is still demonstrated in procedures in which ghrelin increases[80].

Pancreatic polypeptide

There is mounting evidence delineating the role of the vagus nerve in the regulation of the physiology of ghrelin and pancreatic polypeptide (PP). The vagus nerve innervates much of the gastrointestinal tract and helps mediate several hormonal pathways. In humans, blockade of vagal impulses with the use of a non-specific cholinergic antagonist reduces ghrelin secretion[105,106]. Furthermore, administration of exogenous ghrelin to patients with truncal vagotomy after lower esophageal or gastric surgery does not stimulate increased food intake[107]. This suggests that the vagus nerve is involved in the physiological regulation of ghrelin.

PP has structural homology to peptide YY. It is produced in endocrine type F cells in the pancreatic islets and is released into circulation after food ingestion and exercise. PP serves as a regulatory hormone by inhibiting pancreatic exocrine secretion, stimulating glucocorticoid secretion, and modulating gastric acid and gastrointestinal motility. It is secreted at a low basal rate in fasting states and is markedly increased during all phases of digestion[108]. Intact vagal cholinergic reflex circuits are a major regulator of PP secretion. The release of PP is diminished by atropine (a cholinergic antagonist) in normal patients and is completely abolished in patients with a vagotomy[109,110].

PP may be intricately involved in the regulation of food ingestion. Administration of exogenous PP has demonstrated suppression of food intake and inhibition of gastric emptying. After its release, PP has been shown in animal models to have a negative feedback mechanism by acting on receptors in the dorsal vagal complex. Thus, PP has a direct effect on the regulation of vagal input to the stomach and pancreas[108]. Since PP concentrations decrease with inhibition of the vagal cholinergic reflex circuits, however, this suggests that the effect of PP on food intake may be indirect and also regulated through the vagus nerve. After RYGB, PP levels have been shown to decrease as early as one day post-operatively, with subsequent normalization to pre-operative levels one month after surgery. This observation may, in part, be due to vagal dysfunction immediately after surgery with an ensuing return to normal function[111]. A comparison of RYGB with vagal nerve preservation to RYGB with vagal nerve dissection does not reveal any significant differences in the overall long term weight loss after three years[112]. A small study identified higher PP levels after RYGB than in patients with SG, but this finding did not reach statistical significance[113].

CCK

CCK is produced in the I-cells, which are mainly located in the duodenum and proximal jejunum, with small numbers of cells present throughout the rest of the small intestine. It is secreted after food ingestion, especially after meals with high contents of fat and protein[114]. The main functions of CCK are stimulation of pancreatic exocrine secretion, gall bladder contraction, inhibition of gastric emptying, and decreasing food intake[115]. Serum concentrations of CCK rise within 15 min after meals, but have a short plasma half-life of only a few minutes. The anorectic effects of CCK are mediated by its receptors within the central nervous system, which may allow it to operate as a stimulus for satiety[116].

A small preload meal causes a normal physiological response of CCK release with subsequent reduction in food intake and decreased sensations of hunger[117,118]. Parenteral administration of CCK has similar effects by increasing fullness and reducing hunger and food intake, irrespective of whether the patient is lean or obese[119,120]. These findings suggest the possibility that obese patients could have a defect in the secretion of CCK. However, this theory has been refuted by studies showing similar postprandial plasma CCK levels in lean and obese individuals[121,122]. Patients undergoing RYGB do not have statistically significant changes in the post-operative CCK levels[123,124]. After VBG, there were no significant changes in the CCK concentrations in response to a meal on consecutive days before and after surgery[122]. However, when comparing CCK levels of morbidly obese patients three months after VBG to that of lean controls, the peak concentration was significantly higher and required a shortened time period to reach its zenith[125]. These data suggest a possible role of CCK in stimulating satiety and encouraging decreased food intake to help achieve greater weight loss after VBG.

Gut microbiota

The physiology and pathophysiology of the gut microbiota in obesity and in treatment of obesity warrants extensive investigation. There have been preliminary studies examining the role of the gut microbiota in outcomes af-

ter bariatric surgery. This is of course a complex area of clinical research due to differences in concentrations of aerobic and anaerobic bacteria in different regions of the gut and due to differences in the composition of bacteria present in different individuals.

In a study from the Mayo Clinic, Scottsdale[126], nine individuals (3 normal weight, 3 morbidly obese, and 3 after gastric bypass surgery) had extensive studies of their microbial communities. There were 6 major bacterial divisions identified. *Firmicutes* were dominant in individuals with normal weight and morbid obesity, but were decreased after gastric bypass. Morbidly obese individuals had increased numbers of hydrogen-producing *Prevotellaceae* and hydrogen-oxidizing *Archaea*. The authors hypothesized that interspecies hydrogen transfer is an important mechanism for increasing energy update by the human colon in morbidly obese individuals.

A second study from France examined fecal samples from 13 normal weight individuals and from 30 obese individuals (who had samples obtained before and after RYGB)[127]. The major findings included: (1) Bacteria in the *Bacteroides/Prevotella* group were lower in obese individuals compared to normal weight individuals, but increased 3 mo after RYGB; (2) *Escherichia coli* had increased 3 mo after RYGB; (3) Lactic acid bacteria decreased by 3 mo after RYBG; and (4) *Faecalibacterium prausnitzii* was lower in individuals with diabetes mellitus.

The authors hypothesized that components of the gut microbiota adapt to a starvation-like process following RYGB.

A second French study[128] later reported studies of fecal samples and gene expression in white adipose tissue obtained from morbidly obese individuals before and after RYGB. The authors identified enrichment of gut microbiota after RYGB with 37% of increased bacteria being *Proteobacteria*. It was reported that there were increased associations between gut microbiota composition and gene expression in white adipose tissue after RYGB.

Taken together, it is clear that further work is needed to determine how the composition of the gut microbiota and changes in the gut microbiota after bariatric surgery can be used in decision making for the clinical care of morbidly obese individuals who are seeking interventional therapy to induce weight loss.

HORMONES SECRETED BY ADIPOSE TISSUE

Leptin

The hormone Leptin was originally described as important for suppression of food intake in mice. Human Leptin is a 167 amino acids protein. It is produced by adipocytes of mainly white adipose tissue as well as by brown adipose tissue[129]. Leptin is produced in small amounts by multiple organs. The major Leptin receptor is expressed in the hypothalamus, the site of regulation of body weight and the sense of hunger. It has been reported that blood levels of Leptin are paradoxically increased in obese individuals[129].

The potential influence of Leptin on outcomes after bariatric surgery is unclear. It has been reported[129] that Leptin administration compared to placebo (delivered by subcutaneous injections twice daily for 16 wk) in 27 women enrolled at least 18 mo after RYGB revealed that Leptin treatment had no effect on body weights. This result was disappointing since multiple groups[130-132] have reported marked postoperative decreases in blood Leptin levels after SG and RYGB. Further clinical research is therefore required to determine whether Leptin is involved in the weight loss process after these bariatric surgical procedures.

Adiponectin

The hormone Adiponectin is a 244 amino acids protein. Adiponectin is physiologically involved in the regulation of blood glucose levels and fatty acid breakdown or oxidation[133]. It is secreted by adipose tissue, and, interestingly, by the placenta during pregnancy. In adults, there is an inverse relationship between blood Adiponectin levels and body fat percentage. Adiponectin levels may be paradoxically decreased in obese individuals.

Increases in blood Adiponectin levels after bariatric surgery may be important in postoperative insulin sensitivity[133-135]. It has been reported that Adiponectin serum levels increase after RYGB, even in patients with preoperative body mass index as low as 22 kg/m^2[133]. However, investigators in the Swedish Obese Subjects Study have suggested that baseline blood levels of Adiponectin do not predict the treatment benefit of bariatric surgery[136]. Therefore, it is not presently clear how blood levels of Adiponectin could be used to plan future minimally invasive therapy for medically-complicated obesity.

ENDOSCOPY AND THE POTENTIAL FUTURE OF BARIATRIC PROCEDURES

Although bariatric surgery is an effective therapy in the treatment of morbid obesity and has become a cornerstone, it has risks and limitations. Bariatric surgery is the most effective weight loss treatment; however, it is associated with morbidity, mortality, and has a considerable cost. While laparoscopic procedures have reduced the rate of complications, additional advances are actively being sought to further reduce the overall risk and cost. An ideal operation would provide a safe, durable, and reversible procedure with low peri-operative complications and recovery time, without the need for an incision through the abdominal wall. One obvious candidate to help achieve these goals would include endoscopic approaches with interventions performed entirely through the gastrointestinal tract using flexible endoscopes. Not only is this approach becoming more feasible with technological advancements, but it also introduces new categories of therapy.

There are several other types of endoscopic techniques that mimic the anatomic features of bariatric

Table 3 Endoscopic techniques for treatment of obesity

Technique	Proposed mechanisms of action
Intragastric balloon	Increases gastric distension and satiety and delays gastric emptying
Endolumenal vertical gastroplasty	Restriction of food intake and Induce early satiety
Transoral gastroplasty	Restriction of food intake
Trans-oral endoscopic restrictive Implant system	Restriction of food intake
Duodeno-jejunal bypass sleeve	Reduced intestinal digestion and absorption And delayed gastric emptying
Gastroduodenojejunal bypass sleeve	Prevent absorption of nutrients
Aspiration therapy	Reduced presence of available nutrients

surgery. Two broad categories of endoscopic weight loss modalities include restrictive and malabsorptive therapies. Although these categories are overly simplistic and do not explain the hormonal changes that accompany each type of procedure, they have remained prevalent within the literature. Restrictive procedures attempt to decrease the available volume of the stomach with suturing or stapling devices. Malabsorptive procedures prevent food from contacting the duodenum and jejunum and mixing with biliary secretions until more distal segments of the small bowel. Examples of restrictive procedures include intragastric balloon therapy, endolumenal vertical gastroplasty, transoral gastroplasty, and transoral endoscopic restrictive implant systems. Malabsorptive procedures include the duodeno-jejunal bypass sleeve (DJBS) system, which has certain adaptations that may act as a combination of both restrictive and malabsorptive procedures, and mimics many of the hormonal changes seen in bariatric surgery. These endoscopic methods are summarized in Table 3.

Intragastric balloon

The Garren-Edwards bubble (GEB) was the first intragastric balloon widely used in the United States during the 1980s. It was first introduced in 1982 and approved by the Food and Drug Administration in 1985. The GEB was endoscopically deployed in the stomach and expanded using an air insufflation catheter to fill the polyurethane cylinder, which was freely floating in the stomach. It was designed to decrease caloric intake and induce weight loss by increasing gastric distension and satiety and delay gastric emptying[137]. The GEB was less efficacious than bariatric surgery[138] and did not induce statistically significant weight loss in controlled trials. Due to a lack of efficacy and a relatively high cost of this device, it fell out of favor[139,140]. Furthermore, there were several complications including gastric erosions and ulcers, Mallory-Weis tears, small bowel obstruction, and esophageal laceration. Accordingly, the GEB was withdrawn from the market in 1988.

The BioEnterics intragastric balloon (BIB) was first introduced in 1999. It consists of an adjustable silicone elastomer balloon with a spherical shape. The potential complications are similar to that of GEB, but occur less frequently[137]. The Food and Drug Administration has not given approval for BIB use in the United States, but it has been studied extensively worldwide and is approved in many regions including Europe, several South American countries, Australia, and Canada. The prospects of long-term therapy with BIB remain limited as it is usually left in place for only six months. Retrospective data revealed a low complication rate of approximately 2.8% with EWL of 33.9% and improvement in pre-operative co-morbidities in 44.8% of patients[141]. Similar results have been confirmed by several studies validating BIB as an effective and safe short-term treatment of obesity[142,143]. A small prospective study compared weight loss achieved with BIB and laparoscopic AGB. Since BIB is only approved for six month intervals, the device was removed at six months and replaced with an identical, new BIB during the same procedure. Similar results were noted in the two groups after six months; however, at 12 mo, patients with BIB had greater weight loss than laparoscopic AGB. After 18 mo (six months after the BIB had been removed), overall weight loss results were similar as the BIB group had regained some of the lost weight[144].

The long-term outcomes are not as favorable as short-term outcomes with only one quarter of patients maintaining weight loss 2.5 years after intragastric balloon[145]. Thus, some have proposed intragastric balloons as an effective bridge to bariatric surgery. It helps induce pre-operative weight loss to decrease the technical difficulties associated with bariatric surgeries in morbidly obese individuals, and decreases the risk of general anesthesia[146,147]. Furthermore, while it may not change the total weight loss after surgery, pre-operative treatment with intragastric balloon has been shown to reduce intraoperative complications and risk of conversion to open surgery in morbidly obese patients undergoing laparoscopic AGB[148].

Because of this reported unfavorable long-term outcome, other authors have suggested treatment with serial balloons[149]. In a six year study, 83 individuals with BMI > 40 underwent intragastric balloon placement. After balloon removal, a second balloon was placed in all patients after they had regained ⩾ 50% of the weight loss achieved with the initial balloon. A third balloon was placed in 22% of the patients and 1 patient underwent placement of a fourth balloon. At 76 mo of follow up, the mean BMI was 37.6 kg/m^2, demonstrating a modest weight loss. Due to the placement of multiple intragastric balloons, this strategy is less likely to be cost effective.

To address some of the limitations experienced with BIB, the spatz adjustable balloon system (SABS) was introduced. The SABS is composed of a spherical silicone balloon mounted on a catheter placed on one surface. The catheter has two perpendicular loops: one is an easily visible white inflation valve, and the other has a metal chain, which prevents collapse of the balloon and maintains its 7 cm diameter within the gastric lumen to prevent migration beyond the stomach. The device may be removed with a

standard polypectomy snare by ensnaring a blue clasp at the end of the larger, metal chain containing loop. A pilot study was completed with 18 patients and demonstrated 26.4% EWL at 24 wk and 48.8% EWL at 52 wk. Six successful downward adjustments were made to help alleviate intolerances including abdominal pain, nausea, and vomiting. Upward adjustments were made in ten patients due to weight loss plateau resulting in additional weight loss. Several adverse events were experienced causing premature removal of the device in seven patients. This also prompted several improvements in the design of the device to help prevent complications[150].

Despite improvements in the design of the SABS, studies have shown a relatively high rate of adverse events, which remains one of its biggest limitations. Espinet-Coll *et al*[151] experienced adverse events in 15% of the devices used in a study involving 107 patients. Four devices developed a leak and the other 12 developed intolerances requiring early explantation. The intolerances cited include anchor migration in seven patients with two developing duodenal ulcers (one of which was surgically repaired), one gastric fundus ulcer, and four clinical intolerances. One case report was published with a patient experiencing migration of the SABS to the jejunum causing obstruction and the patient presenting with abdominal pain, nausea, and vomiting 8 mo after implantation of the device. Exploratory laparotomy was performed with removal of the SABS through a small enterotomy[152]. In a direct comparison of SABS and BIB in a case control study, both devices were well tolerated with similar weight loss after 12 mo. However, the SABS group experienced complications in 17.5% compared to only 2.5% of those with BIB. Device migration was noted in 10% of the SABS group and 15% of patients had to have it removed prematurely[153]. The SABS could potentially be used in the same manner as some studies have demonstrated with BIB. The device may be removed one year after the initial procedure with subsequent placement of a new SABS device. This type of approach could allow placement for up to two years[153]. Although preliminary studies are encouraging, the lack of long-term efficacy and concerns about the overall safety necessitate additional research and possibly further improvements in the design of the device prior to its widespread use.

Another potential alternative system has been developed by ReShape Medical. The ReShape Duo has been used in Europe for over 2 years. This devise consists of two connected but separate balloons with a total volume of 900 mL. In one small study, three sites randomized 21 patients to a dual balloon system for 24 wk, while 9 patients treated with diet and exercise alone served as the control group[154]. These 30 patients included 26 women and 4 men with an age range of 26 to 59 years-old. At 24 wk, the mean excess weight lost averaged 32% in the dual balloon group and 18% in the control group. Twenty four weeks later, the dual balloon group had maintained 64% of their weight loss. In the dual balloon group, the treatment-related complications included severe nausea in 4 out of 21 patients (19%), gastritis in 1 patient, and transient hypoxia in 1 patient during removal of the balloon devise. No long term study results are yet available for review.

Endolumenal vertical gastroplasty

Initial studies of endolumenal treatments for gastroesophageal reflux disease (GERD) introduced endoscopic suturing devices developed to create tissue placation using adjacent tissue folds[155]. This system was marketed as the Bard EndoCinch Suturing System and while it has good short-term outcomes in the treatment of GERD, the long-term results show diminishing efficacy over time[156,157]. The possibility of its therapeutic potential has been extended to treatment of obesity. Endolumenal vertical gastroplasty (EVG) was completed by using a continuous suture pattern to create a narrow, tubular shaped stomach to provide restriction of food intake and to cause early satiety. The underlying hormonal effects of this procedure are unknown as they have not yet been studied. The average procedure time for EVG is approximately 45 min with minimal complications and no serious adverse events. EVG provided outcomes comparable to bariatric surgery with 58.1% EWL at one year[158].

A separate device was developed by Bard and named the restore suturing system. This is a single-intubation, multi-stitch device placed at the end of a standard endoscope. An initial pilot study revealed the feasibility and safety of this procedure, but the overall procedure time took an average of 125 min[159]. A one year follow-up revealed only modest decreases in weight, BMI, and waist circumference with a mean of 27.7% EWL. Furthermore, endoscopy at one year found a partial or complete release of plications in 72% of patients[160]. Despite adequate short-term outcomes with EVG, there is a lack of data showing the long-term durability. Given the results noted with the Bard EndoCinch Suturing System in the treatment of gastroesophageal reflux and the limited data about the findings with the restore suturing system, one might expect the efficacy to further decline in longer-term studies.

Transoral gastroplasty

An endolumenal procedure termed transoral gastroplasty (TOGA) was developed by Satiety Inc. and involves endoscopic stapling of the lesser curvature of the stomach to create a restrictive pouch. The pilot study for this device did not have any serious adverse events and had good results with 24.4% EWL at six months post-treatment. The study did note gaps in the original staple line in 62% of patients[161]. The TOGA system was subsequently improved by developing overlapping staple lines to avoid gaps from forming and a small follow-up study involving 11 patients was conducted to test the new advancements. This study confirmed the safety of the procedure and resulted in 46.0% EWL at six months[162].

Although there have been good short-term outcomes, there is limited data about the long-term effects.

Outcomes measured at one year in a study with 53 patients revealed a total of 38.7% EWL. Complications were reported with one patient developing respiratory insufficiency requiring mechanical ventilation and another with asymptomatic pneumoperitoneum. Furthermore, 23 of the patients developed gaps in the staple line seen on endoscopy[163]. Another recent study involving a comparison of TOGA to RYGB and BPD revealed good outcomes for a select group of patients with TOGA after two years. Although RYGB and BPD had better overall results in the reduction of BMI, the TOGA patients with a lower initial BMI had higher reductions in their BMI than patients with RYGB and BPD[164]. At this time, the TOGA system is not used within the United States. Clinical trials did not reach set targets and research and development of the product was halted indefinitely due to a lack of approval from the United States Food and Drug Administration.

A variation of TOGA was recently described in a clinical study performed at the Mayo Clinic, Rochester by one of us (Gostout CJ) using the commercially available endoscopic suturing device, Overstitch. This novel procedure accomplishes endoscopic gastric volume reduction in a similar fashion to SG. It introduces a series of closely spaced, full-thickness, interrupted sutures through the gastric wall resulting in volume-reducing plications extending from the gastric antrum to the gastroesophageal junction. A pilot study with four patients demonstrated the technical feasibility of this endoscopic procedure without any intra-operative or serious post-operative adverse events[165].

Two additional intralumenal suturing systems have been recently reported. Espinos and associates have described a per-oral system that is designed to place transmural plications in the gastric fundus and distal gastric body using specialized suture anchors[166]. Their clinical study included 45 patients (76% females). A mean of 8.2 plications were placed in the fundus and a mean of 3.0 plications were placed in the distal gastric body. At 6 mo follow up, patients had lost a mean of 49.4% of their excess body weight with no mortality and no operative morbidity reported. This interesting report will require verification of the authors' results in additional research centers and long-term follow up of weight loss to confirm the utility of this technique.

In a multicenter report, there has been a recent, partial description of a new transoral suturing device developed by SafeStitch Medical Inc, Miami, Florida, United States[167]. The authors describe four obese patients who underwent proximal gastric placement of gastroplasty sutures after mucosal excisions of gastric tissue. It was reported that at 2 year follow up, the patients had excess body weight lost that ranged from 0% to 68%. This study is quite limited in that it is a small clinical trial with highly variable results. In addition, the physiological basis for the placement of gastroplasty sutures and the mechanisms involved in weight loss are not well defined.

Despite the promising short-term results of TOGA and its variations, long-term results are not yet available. The durability and extent of weight loss remain in question and thus far, these procedures have not demonstrated clear advantages in efficacy over surgery. These procedures may be advantageous in a select subgroup of morbidly obese patients, but further studies are necessary to elucidate the long-term outcomes when compared to other surgical and non-surgical options.

Trans-oral endoscopic restrictive implant system

The trans-oral endoscopic restrictive implant system (TERIS) was developed by BaroSense Inc. and introduced as a new endoscopic therapy for the treatment of obesity[168]. This procedure involves placement of a restrictor with a 10 mm central channel for food passage at the gastric cardia, creating a restrictive pouch. This is considered a permanent implant, but may be removed or modified if necessary. A prospective, observational study was completed to evaluate the short-term safety and efficacy of TERIS. Of a total of 13 patients included in this study, three of them experienced serious adverse events with one developing gastric perforation requiring procedural reversal and laparoscopic treatment and two others developing pneumoperitoneum. The percentage of EWL was 22.2% after three months[169]. While the procedure is feasible and weight loss and improvement in quality of life measures are comparable to restrictive bariatric procedures, the safety profile remains a concern. Technical improvements and long-term data are necessary to make TERIS an effective option in the arsenal of treating obesity. Completion of multi-center feasibility studies would be important in order to evaluate adverse events relating to the procedure and device and the associated overall weight loss. At this time, it does not appear that clinical research studies utilizing TERIS will be proceeding.

DJBS

Developed as the EndoBarrier by GI Dynamics, Inc. (Lexington, Massachusetts, United States), the DJBS is an endoscopically implanted, removable, and impermeable fluoropolymer sleeve with a nitinol anchor. The device is deployed into the duodenal bulb under fluoroscopic guidance during endoscopy and extends 60 cm into the small bowel. It is intended to mimic a bypass procedure without the risks and effects associated with surgery. It does not allow food to contact the mucosa of the duodenum and upper portions of the jejunum. It also prevents food from mixing with biliary and pancreatic secretions until more distal segments of the jejunum. The device was initially studied in porcine models without significant adverse events[170], and was later successfully deployed in the first human subject[171]. An open-label, single-center, prospective study including 12 patients revealed good outcomes with 23.6% EWL after 12 wk. Two patients had explantation of the device after nine days secondary to poor placement and there were no severe device-related events reported[172]. In a larger, multicenter, randomized clinical trial involving 41 patients with 30 undergoing

duodeno-jejunal bypass sleeve placement, there were no adverse events. The device could not be implanted successfully in 4 patients, but patients with the device experienced 19% EWL after 3 mo compared to 6.9% EWL in control patients[173].

Another variation of the duodeno-jejunal bypass sleeve has been tested using a restrictor orifice to add a restrictive component. This device, also endoscopically implanted into the duodenal bulb, has a 4 mm orifice to slow gastric emptying. The device was first tested in porcine models and after safety was established, was used in a pilot study including 10 patients. This open-label, single-center trial investigated the modified duodeno-jejunal bypass sleeve with restrictor orifice and found the results of duodenal exclusion and delayed gastric emptying to be additive. Patients had an average of 40% EWL after just 12 wk, which compared favorably to results with the unmodified duodeno-jejunal bypass sleeve after 12 wk. The gastric emptying rate was measured by scintigraphy and noted to be significantly reduced in all patients with the device. There were no clinically significant adverse events, but a majority of patients experienced abdominal pain, while others had nausea and vomiting. These symptoms lead to seven patients requiring balloon dilation of the restrictor orifice[174]. Currently, there are no available data for the long-term outcomes for this device.

The duodeno-jejunal bypass sleeve has also been tested in obese patients with type 2 diabetes mellitus. The study included 22 patients and was an open-label trial lasting 52 wk. Only 13 patients completed the entire duration of the study. Reasons for explantation included device migration in three patients, gastrointestinal bleeding in one patient, abdominal pain in two patients, and unrelated reasons in the remaining three patients. The duodeno-jejunal bypass sleeve improved the overall glycemic status of the 13 patients that completed the entire study and the reported EWL was 39.0%[175]. The duodeno-jejunal bypass sleeve device already has approval and is being used in several countries. A large, randomized, sham-controlled clinical trial is underway in the United States. The long term results of the duodeno-jejunal bypass sleeve remain in question as there is no long-term data. Furthermore, the device is only approved for a short duration of 12 mo and placement for longer intervals has not yet been tested.

Gastroduodeno-jejunal bypass sleeve

A novel device was developed by ValenTx Inc designed to mimic the restrictive and malabsorptive aspects of RYGB without the risks of bariatric surgery. An endolumenal, endoscopic gastroduodeno-jejunal bypass sleeve was proposed to induce short-term weight loss and improvements in co-morbidities. This sleeve is a 120 cm long fluoropolymer deployed through the pylorus with a toposcopic delivery technique with flow and pressure monitoring. Fluoroscopic guidance is used to ensure proper deployment through the duodenum and into the proximal jejunum. The device is then secured at the gastroesophageal junction using endoscopic and laparoscopic techniques. Explantation may be completed with an endoscopic retrieval method. The resulting gastroduodeno-jejunal bypass sleeve consequently prevents absorption of nutrients in the stomach, duodenum, and jejunum and delivers food directly from the esophagus to the small bowel.

An initial prospective, single-center trial included 24 patients. Two patients were excluded pre-procedurally and the device was successfully placed in 22 patients. The 12 wk study was completed by 17 of the patients, who had a reported 39.7% EWL. There were no serious adverse events experienced and the main reason for early explantation in five patients was pain with swallowing[176]. Further development of this device may eliminate laparoscopic visualization and allow the procedure to be performed entirely endoscopically. Although the early results are promising with weight loss results similar to those reported with RYGB and SG, further short-term and long-term data is needed to establish the clinical efficacy of the gastroduodeno-jejunal bypass sleeve in the treatment of morbid obesity.

Aspiration therapy

There is an endoscopic weight loss technique termed "Aspiration Therapy" which is not designed to mimic the physiological effects of bariatric surgery[177]. In this endoscopic technique, a specialized tube called as aspiration tube or A-Tube (Aspire Bariatrics, King of Prussia, Pennsylvania, United States) is placed percutaneously, leaving both an intragastric portion with holes to permit aspiration as well as a skin port. Ten to 14 d after A-Tube placement, individuals in this single center trial were instructed to aspirate their gastric contents 20 min after any breakfast, lunch or dinner that included more than 200 kcal. In this trial involving 11 subjects, ten individuals completed the first year of the study. These ten subjects lost a mean of 49% of their excess body weight at 1 year. Seven of these 10 subjects were able to maintain their weight loss at 2 years after enrollment in this study. This of course is a small, single center clinical study. More extensive validation will be required for assurance that the subjects are not simply being allowed to or encouraged to develop a potentially harmful eating disorder.

OTHER PROCEDURES

Other techniques have been proposed to induce weight loss by attempting to alter normal physiology. These techniques, as shown in Table 4, are summarized in the below section.

Electrical stimulation

Several animal and human studies have been completed on the effects of electrical stimulation of the stomach and intestines as a mode of therapy for morbid obesity. Animal studies have shown promising results with electrical stimulation of the intestines, which causes gastric

Table 4 Miscellaneous techniques for treatment of obesity

Technique	Proposed mechanisms of action
Electrical stimulation	Reduced gastric accommodation and Delayed gastric emptying
Intragastric botulinum toxin injections	Prolonged gastric emptying time and Reduced maximal gastric capacity
Natural orifice translumenal Endoscopic surgery	Transluminal access to intra-abdominal Structures

relaxation, delays gastric emptying, and accelerates intestinal transit normally slowed by the ileal brake[178-180]. In humans, gastric electrical stimulation was found to be a safe and feasible therapy for morbid obesity. Several trials showed significant amounts of weight loss due to reduced gastric accommodation, delayed gastric emptying, and increased intestinal transit[181-184]. Direct comparisons of these studies is difficult as several different approaches have been attempted including laparoscopic and endoscopic placement of electrodes. Additionally, the exact placement varies with some studies placing electrodes in the distal stomach and others in the duodenum. Although these results have been encouraging, many questions remain about this modality of therapy and its long-term results.

Another area of active research has been vagus nerve stimulation (VNS). As previously noted, the vagus nerve plays an important role in the hormonal regulation pathways involved with satiety and short-term regulation of food intake. Bodenlos *et al*[185] demonstrated that depressed patients experience a significant change in ratings of cravings for sweet foods with acute activation of a VNS device. Similarly, providers administering treatments with cervical VNS for adjunctive therapy of severe, treatment-resistant depression noted significant weight loss proportional to the initial BMI, with more severe obesity yielding greater amounts of weight loss[186]. While truncal vagotomy has also successfully been used in the treatment of obesity[187,188], its effects may not be maintained over time, possibly owing to the development of collateral innervations[188].

A new therapy termed VBLOC for Vagal BLOCking therapy was developed for the treatment of obesity using intermittent intra-abdominal vagal blocking with high-frequency electrical currents. During a laparoscopic procedure, electrodes are placed on the anterior and posterior vagal trunks near the gastro-esophageal junction. A neuroregulator is placed subcutaneously and an external controller is used to program the device. This procedure does not require any anatomical modification or tissue compression within the gastrointestinal tract. An open-label, multi-centered trial was conducted to assess the feasibility, safety, and efficacy of VNS. Patients had a mean EWL of 7.5%, 11.6%, and 14.2% at four weeks, 12 wk, and six months, respectively. There were no device-related serious adverse events and patients were noted to have decreased caloric intake, early satiety, reduced hunger, and decreased plasma PP levels[189].

The EMPOWER study was a randomized, prospective, double-blinded trial that included 294 patients from 15 centers. All patients had an implanted VBLOC and were randomized to treated or control groups for 12 mo. Device-related complications were observed in 3% of patients with nearly half due to preexisting conditions. After 12 mo, the EWL for the treated and untreated groups were 17% and 16%, respectively. There was no statistically significant weight loss between the two groups. However, the authors suggest that electrical safety checks of the equipment deliver a low charge for electrical impedance, safety, and diagnostic checks, which may have contributed to the weight loss observed in the control group[190]. Despite promising results seen in the initial studies, the most extensive study to date was unable to demonstrate a clear advantage with the use of VBLOC. Given the favorable safety profile and ease of placement of the device without alteration of the gastrointestinal anatomy, further studies are needed to determine its long-term efficacy and feasibility in the treatment of obesity.

Intragastric botulinum toxin injections

Botulinum toxin (BTX) acts to inhibit acetylcholine release at the neuromuscular junction and causes a local paralysis. This agent has been studied in the treatment of obesity with injections into the wall of the stomach to inhibit motility and delay gastric emptying. Initial experiments in animal models showed that injection of BTX into the gastric antrum caused a reduction in food intake and caused a reduction in body weight[191]. Initial human studies showed the overall safety of the procedure with no clinically significant adverse events. However, the overall outcomes were mixed. Some patients reported early satiety, but BTX injections did not provide a clear benefit for weight loss[192-194]. In double-blinded, randomized, controlled trials with BTX injections in the gastric antrum, the effects of gastric emptying were variable. Although some patients reported a reduced appetite, the amount of weight loss was not statistically significant[195,196].

A slight variation was attempted by Foschi *et al*[197] with intraparietal endoscopic administration of BTX into the gastric antrum and fundus. This double-blinded controlled study found a prolonged gastric emptying time and reduced maximal gastric capacity for liquids. Patients treated with BTX had significantly greater amounts of weight loss after eight weeks. Given the minimal adverse events noted in studies and the widespread availability, BTX has the potential to have a large role in the treatment of obesity. However, additional investigation into the sites of BTX injection and the optimum dosage are necessary. One major limitation with BTX is its relatively short duration of action, which is approximately three to six months. Furthermore, data regarding long-term outcomes is lacking and additional studies may be warranted.

NOTES

NOTES procedures provide the obvious advantage to patients of a surgical procedure without the externally

visible incision and resultant scars. There are several advantages to performing traditional surgical procedures with endoscopic access through natural orifices, including reduced complications and duration of hospitalization and a faster recovery period. Although the endoscope has traditionally been used for treatments within the gastrointestinal lumen, recent ventures have explored translumenal access to intra-abdominal structures. Technological advancements in endoscopy combined with efforts to find less invasive, safer therapies in the field of bariatrics have cultivated endolumenal bariatric surgery.

The basic principles of NOTES began with the description of the first percutaneous endoscopic gastrostomy tube insertions[198]. Multiple novel experimental techniques were described in animal models as this new form of minimally invasive surgery became more feasible[199-205]. With continued development of the technique, one of the main difficulties encountered was achieving adequate spatial orientation. Hybrid versions with the use of standard laparoscopic vision together with endoscopic surgical procedures were first tested for feasibility in animal models[206] and later used in animals to perform more technically advanced procedures including SG[207,208].

Using a hybrid technique with a transvaginal and transgastric approach, the first RYGB was described in a human cadaver to demonstrate the feasibility of the procedure[209]. A similar study by Hagen *et al*[210] attempted RYGB in cadavers, but they experienced several obstacles. Difficulties included bowel manipulation and measurement, tissue dissection, stapler manipulation, and anvil docking. Furthermore, the total procedure time was approximately 6-9 h. While the authors found the procedure to be feasible, they noted a lack of proper instrumentation as a major barrier. The hybrid form of NOTES was used by Ramos *et al*[211] in the first description of SG performed in four patients using a transvaginal approach. The total operative time was 90 to 100 min and the procedure was shown to be feasible and safe with no short-term complications reported. In order to enhance the ability of the operators in helping to navigate the instruments, an image registration system has been evaluated in cadavers. Operators were better able to reach their intended targets as this system provided them with enhanced navigation without the need for previous training or knowledge of the system[212]. However, this system has not been widely tested and is not currently used in conjunction with NOTES.

There are several limitations to the growth and expansion of NOTES, which initially generated high expectations. Advanced spatial and endoscopic skills are required by practitioners to apply new techniques and learn to operate new technology. Additionally, the advancement in technology in production of the next generation of endoscopic tools in the United States has been slowed by economic considerations and stringent regulatory standards[213]. With the increasing interest and growing number of studies on NOTES, the Society of American Gastrointestinal and Endoscopic Surgeons and the American Society for Gastrointestinal Endoscopy assembled a Joint Committee to review ongoing issues involving NOTES. As a part of this effort, the Joint Committee formed an organization called the Natural Orifice Surgery Consortium for Assessment and Research (NOSCAR). In their most recent publication, NOSCAR notes that a major roadblock to further advancement of NOTES remains within the regulatory and reimbursement arenas. Innovative technology to enable the performance of NOTES is mostly being developed by small startup companies that cannot withstand regulatory and reimbursement difficulties of the industry. Another challenge considered by NOSCAR is the difficulty in defining the optimal human procedures for the first widespread application of NOTES. The best potential human applications are considered to be transanal/vaginal colorectal surgery, endolumenal myotomy for achalasia, and staging peritoneoscopy for GI malignancies. Despite the promising research and growth of NOTES in bariatrics, applications to this field are not currently considered as one of the most promising areas of growth by NOSCAR[214].

CONCLUSION

Obesity and its associated co-morbidities are on the rise worldwide and have reached epidemic proportions. To help treat this growing and devastating disorder, the field of bariatrics has been evolving and growing over the past several decades. Surgical procedures have been developed and refined to help manage obesity, and over time, the safety and efficacy of bariatric surgery has improved. Due to the significant benefits and minimal associated risks, bariatric surgery is now the preferred modality of therapy for morbidly obese individuals, especially those with co-morbidities.

Surgical options for medically-complicated obesity currently are supported by the best available data and generally provide the best outcomes. However, attempts to replace invasive techniques, with their related morbidity and mortality, are the driving factors behind studies of newer, minimally invasive procedures. Our therapeutic armamentarium continues to expand for treatment of morbid obesity and its medical complication as new research is completed and novel techniques are assessed. Preliminary results in several of these areas are promising and provide patients and practitioners with a potential future array of options and modes of therapy, yet many questions remain regarding the safety, efficacy, and durability of these new procedures. With continued research and additional technological advancements, new safe and effective minimally invasive treatment options appear to be on the horizon.

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REVIEW

Stool characteristics of infants receiving short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides: A review

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Abstract

Human milk is considered to be the optimal source of infant nutrition. Some of the benefits of breastfeeding have been ascribed to human milk oligosaccharides (HMO). For instance, HMO can affect faecal characteristics such as stool consistency and stool frequency. Such effects on stool characteristics can be beneficial for young infants as hard stools and even constipation is common in that age group. Prebiotics in infant milk formulas have been introduced to exert similar functionalities. A specific mixture of prebiotics consists of a combination of short chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) in a ratio of 9:1. This specific mixture has been developed to closely resemble the molecular size composition of HMO. Many studies have been done with scGOS/lcFOS, and indicators for digestive comfort have often been included as secondary outcomes. This review summarizes the effects of scGOS/lcFOS (9:1) on stool consistency, stool frequency and transit time in healthy term and preterm infants. In several of the studies with scGOS/lcFOS in a ratio of 9:1 in infant milk formulas, positive effects of this mixture on stool characteristics such as stool consistency and stool frequency were observed. As stool consistency was shown to be correlated to whole gut transit time, scGOS/lcFOS can be hypothesised to have a role in reducing the risk of constipation.

Key words: Short-chain galactooligosaccharides; Long-chain fructooligosaccharides; Stool consistency; Stool frequency; Infants

Core tip: In several clinical trials with a specific mixture of short-chain galacto-oligosaccharides and long-chain fructooligosaccharides in a ratio 9:1 in infant milk formulas, positive effects were observed on stool consistency and stool frequency. This specific mixture of short chain galacto-oligosaccharides and long-chain fructo-oligosaccharides may therefore have a role in reducing the risk of constipation.

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STOOL CHARACTERISTICS AND CONSTIPATION IN INFANCY AND CHILDHOOD

Childhood constipation is a common problem, with a

prevalence ranging from 0.7% to 29.6%[1]. It is mentioned to be the main reason for a paediatric outpatient visit in 3% of all cases[2] and for 30% of the visits to paediatric gastroenterologists[3,4]. Symptoms of constipation start to occur during the first year of life in about 40% of children with functional constipation[5], In the past, infants on standard infant formula have been reported to suffer from hard, infrequent stools and even from constipation more often than infants receiving human milk[6-8]. This difference has partly been ascribed to human milk oligosaccharides (HMO), which are present in human milk in levels of approximately 1 g/100 mL[9].

EFFECTS OF HMO ON STOOL CHARACTERISTICS

HMO are prebiotic oligosaccharides[10], which are not hydrolysed by digestive enzymes in the small intestine[11,12]. In the colon, undigested substrates can be used by colonic bacteria: the intestinal microbiota. The intestinal microbiota is dependent on the provision of substrates for their growth. For instance, non-digestible carbohydrates such as dietary fiber and dietary oligosaccharides that reach the colon intact can stimulate the growth of colonic bacteria, resulting in an increase in bacterial mass. HMO is non-digestible oligosaccharides that reach the colon intact, and can be used as substrate for the intestinal microbiota[12-14]. As bacteria in the colon have a preference for specific types of substrates, the provision of a specific type of substrate in the form of HMO will result in selective fermentation by intestinal bacteria, which can change the composition of the intestinal microbiota. In this case, fermentation of the non-digestible carbohydrates from human milk is associated with selective growth of Lactobacillus species and Bifidobacteria[12,15]. As end-products of fermentation, lactate and short-chain fatty acids (SCFA), such as acetate, propionate and butyrate are produced. Lactate and SCFA can then be absorbed and used as an energy source for the host, but within the colon, SCFA have also been shown to have specific effects, such as being an energy source for the colonic mucosa, to have trophic effects on the intestinal mucosa, to stimulate mucosal blood flow and oxygen uptake and to affect sodium and water absorption [16]. Furthermore, human milk oligosaccharides have been suggested to increase mineral absorption[17,18].

As mentioned before, stool characteristics of infants who recieved human milk have in the past been reported to differ from infants on standard infant formula[6-8]. This difference has partly been ascribed to HMO. Several mechanisms can explain these effects. First, an increase in microbial mass due to the fermentation of the oligosaccharides can increase the faecal water content, which can results in softer stools. Second, the selective fermentation and growth of Lactobacillus species and bifidobacteria[19,20] and the subsequent production of SCFA can increase the water content of the faecal mass , but SCFA may also stimulate gastrointestinal motility, either by being used as energy source for colonic epithelial cells, or by inducing phasic and tonic contractions in circular muscle[21-25]. Third, as HMO are specific types of dietary fiber, they can be hypothesized to bind water and thereby increase the water content of the faecal mass[22].

EFFECTS OF SCGOS/LCFOS ON STOOL CHARACTERISTICS

In the last decade, several types and mixtures of non-digestible oligosaccharides have been used in infant formulas, such as galacto-oligosaccharides, fructo-oligosaccharides, polydextrose, and pectin hydrolysates. Although the compositional complexity of HMO cannot be mimicked completely today, a specific mixture of 90% short-chain galacto-oligosaccharides (derived from lactose, degree of polymerization 3-8) and 10% long-chain fructo-oligosaccharides (inulin extracted from chicory roots, average degree of polymerization > 23) (scGOS/lcFOS) was developed which closely resembles the molecular size distribution of HMO[9,26]. Many studies have been done with this mixture and most of these studies focused on the beneficial effects of scGOS/lcFOS (9:1) on the development of the intestinal microbiota and the maturation of the immune system and measured stool characteristics as secondary outcomes[27]. However, like HMO, fermentation of non-digestible oligosaccharides can potentially benefit faecal characteristics[28]. A review of the colonic effects of all different types of non-digestible oligosaccharides is beyond the scope of this paper. The current review specifically addresses the effects of a mixture of scGOS/lcFOS on faecal characteristics.

In most of the studies with scGOS/lcFOS, tolerance to infant formulas and stool characteristics have been taken into account as secondary outcome outcomes. Only a small number of studies have specifically addressed the effects of non-digestible oligosaccharides on stool characteristics as a primary focus. Furthermore, the different stool characteristics have not been measured with the same methodology, which makes it difficult to compare the outcomes of these studies systematically. However, as these measures have been taken into account in several of these studies, these effects and the potential mode of actions in the individual studies described below. Table 1 summarizes the studies that included effects of a specific mixture of scGOS/lcFOS on stool characteristics.

STOOL FREQUENCY AND STOOL CONSISTENCY

In a study by Costalos *et al*[29] an infant milk formula with 0.4 g/100 mL scGOS/lcFOS (9:1) was compared to a control formula without scGOS/lcFOS in term infants for 10 wk. Stool characteristics were included as secondary outcomes and data were obtained from a 5-d diary at the end of the intervention period. Effects on stool

Table 1 Summarizes the studies that included effects of a specific mixture of short chain galacto-oligosaccharides and long-chain fructo-oligosaccharides on stool characteristics

Author	Age group	Dose	*n*	Outcome	Result in scGOS/lcFOS group
Costalos 2008	Term	0.4 g/100 mL	140 (2 groups)	Stool frequency	Statistically significant increase after 10 wk of intervention
				Stool consistency	Statistically significant softer stools after 10 wk of intervention
Moro 2002	Term	0.4/0.8 g/100 mL	90 (3 groups)	Stool frequency	Statistically significant increase after 4 wk of intervention
				Stool consistency	Statistically significant softer stools after 4 wk of intervention
Veereman 2011	Term	0.8 g/100 mL	76 (5 groups)	Stool frequency	No statistically significant differences
				Stool consistency	Statistically significant softer stools after 2 and 4 wk of intervention
Bisceglia 2009	Term	0.8 g/100 mL	76 (2 groups)	Stool frequency	Statistically significant increase throughout 4 wk of intervention
				Stool consistency	Not measured
Boehm 2002	Preterm	1.0 g/100 mL	42 (3 groups)	Stool frequency	Statistically significant increase after 4 wk of intervention
				Stool consistency	Statistically significant softer stools after 4 wk of intervention
Mihatsch 2006	Preterm	1.0 g/100 mL	20 (2 groups)	Stool frequency	No statistically significant differences
				Stool consistency	Results not provided
				Viscosity	Statistically significant lower viscosity after 2 wk of intervention
Modi 2010	Preterm	0.8 g/100 mL	150 (2 groups)	Stool frequency	No statistically significant differences
				Stool consistency	No statistically significant differences

scGOS/lcFOS: Short chain galacto-oligosaccharides and long-chain fructo-oligosaccharides.

consistency were rated on a 5-point scale, ranging from 1 = watery and 5 = hard. In this study, a statistically significant higher stool frequency and a statistically significant lower stool consistency (softer stools) were observed with the formula with scGOS/lcFOS when compared to the control formula. After 10 wk of intervention, the median number of stools per day was 1.9 and 1.6 in the scGOS/lcFOS (9:1) group and the control group respectively ($P = 0.031$). Stool consistency scores were 3 and 3.1 ($P = 0.026$) in the two groups respectively).

Moro *et al*[30] performed a study in term infants that received an infant milk formula with 0.4 g/100 mL or 0.8 g/100 mL scGOS/lcFOS (9:1) or a control formula without scGOS/lcFOS (9:1) for 28 d. In this study, stool characteristics were also included as secondary outcomes at baseline and after 28 d of intervention. Again, stool consistency was rated on a 5-point scale ranging from 1-watery to 5-hard. A statistically significant lower stool consistency (softer stools) and a statistically significant higher stool frequency were observed in infants that received the formula with 0.8 g/100 mL scGOS/lcFOS when compared to infants that received the control formula. At the end of the intervention, the median number of stools per day was 2 for both groups ($P < 0.01$), and stool consistency scores were 2.3 and 4 in the scGOS/lcFOS (9:1) group and the control group respectively ($P < 0.0001$).

In a study of Veereman-Wauters *et al*[31] a statistically significant lower stool consistency was observed in term infants after 2 and 4 wk of intervention with a formula with 0.8 g/100 mL scGOS/lcFOS (9:1) when compared to a control group without scGOS/lcFOS. Stool characteristics were included as secondary outcomes and were recorded in 3-d diaries at baseline, and after 2 and 4 wk of intervention. Stool consistency was measured on a 4-point scale ranging from 1-watery to 4-hard. Mean stool consistency scores were approximately 1.9 in the scGOS/lcFOS (9:1) group and 2.4 in the control group. In this study, a human milk reference group was included. In that group, the mean stool consistency was 1.3. No statistically significant differences were observed in stool frequency.

Bisceglia *et al*[32] followed a group of 36 term infants from birth until 28 d of life. The infants received a formula with 0.8 g/100 mL scGOS/lcFOS (9:1) or a control formula. Stool frequency was included as a secondary outcome. Stool frequency was recorded throughout the study, and a significantly higher number of stools were recorded in the scGOS/lcFOS (9:1) group when compared to the control group (3.4 and 1.7 stools per day, respectively, $P < 0.001$). In this study, stool consistency has not been recorded.

The studies summarized above focused on effects of scGOS/lcFOS in term infants, but studies in preterm infants show similar effects. In preterm infants, gastrointestinal tolerance to formula feeding is an important aspect, and enteral feeding can be associated with a disturbed gastrointestinal passage. One of the studies with scGOS/lcFOS in preterm infants specifically focused on stool characteristics as a primary outcome. In this study, Mihatsch *et al*[33] observed statistically significant effects of an infant milk formula with 1.0 g/100 mL scGOS/lcFOS (9:1) on stool viscosity and gastrointestinal transit time, but did not demonstrate an effect on stool frequency after 14 d of intervention in preterm infants. Stool viscosity and gastrointestinal transit time were the primary outcomes in this study. Stool characteristics were recorded throughout the study. Stool consistency was shown as a percentage of each type of consistency (hard, formed, soft, mushy or watery) against all stools throughout the study. A higher proportion of soft stools were observed in the infants that received scGOS/lcFOS (9:1) when compared to infants receiving a control formula without scGOS/lcFOS, but no information on the statistical significance is provided in the paper. The median stool viscosity (measured as extrusion force) was significantly lower in the scGOS/lcFOS group when compared to the control group, with a level of 31.8 mol/L and 157.5 mol/

L in the two groups respectively ($P = 0.006$). In addition, a shorter transit time (12 h) was observed in preterm infants that had received a formula with scGOS/lcFOS, when compared to infants who had received a control formula (25.6 h)[33]. In this study, the difference in the change in transit time between the groups from baseline to day 14 was statistically significant (6 h decreased transit time in the scGOS/lcFOS group, and a 9.1 h increased transit time in the control group). The total number of stools did not show a statistically significant difference, but there was a trend toward a higher stool frequency in the scGOS/lcFOS (9:1) group when compared to the control group (3.6 and 2.6 respectively ($P = 0.059$).

Boehm *et al*[34] showed a statistically significant lower stool consistency (softer stools) and a statistically significant higher stool frequency in preterm infants receiving an infant milk formula with 1.0 g/100 mL scGOS/lcFOS (9:1) when compared to infants receiving an infant milk formula without scGOS/lcFOS. In this study, stool characteristics were included as secondary outcomes and data were collected via questionnaires. After 28 d of intervention, a mean number of stools of 2.2 and 1.3 in the scGOS/lcFOS (9:1) and control groups, respectively, was observed ($P = 0.0079$), and a mean stool consistency score of 2.7 and 3.5 in both groups respectively ($P = 0.0102$, mean score from a 5-point scale ranging from 1-watery to 5-hard).

Modi *et al*[35] did not find statistically significant differences in stool frequency and stool consistency between a scGOS/lcFOS (9:1) group (0.8 g/100 mL) and a control group of preterm infants. The intervention lasted from preterm birth until hospital discharge, for a maximum of 12 wk. Stool characteristics were included as secondary outcomes. In this study, a statistically significant effect may not have been observed as stool consistency scores were not based on diary data, but on a single observation of the hospital staff[35]. Furthermore, in this study, formula was supplied in addition to human milk, and the number of children exclusively receiving formula was very low (15%). As human milk may already have a significant effect on stool characteristics, the effect of addition of a limited amount of formula with or without scGOS/lcFOS may not have been visible.

POSSIBLE MECHANISMS OF ACTION

The potential beneficial effects of scGOS/lcFOS on stool characteristics can be mediated by the increase in microbial mass, selective fermentation and production of SCFA, stimulation of gastrointestinal motility, or the direct effects of the provided dietary fibers in the form of scGOS/lcFOS.

The specific mixture of scGOS/lcFOS (9:1) is a mixture of oligosaccharides that is not digested in the gastrointestinal tract and is available for fermentation in the colon[36,37]. Indeed, this mixture has been shown to increase the total concentrations and the individual concentrations and percentages of bifidobacteria and lactobacilli in the colon[29-31,34,36,38-42]. For instance, in the study by Moro *et al*[30] levels of bifidobacteria increased after 28 d of intervention to 9.3 CFU/g faeces in infants who received a formula with 0.4 g/100 mL scGOS/lcFOS, and to 9.7 CFU/g faeces in infants who received a formula with 0.8 g/100 mL scGOS/lcFOS. In the control group, a level of bifidobacteria of 7.2 CFU/g faeces was observed. In a study by Scholtens *et al*[43] intervention with infant milk formulas with 0.6 g/100 mL resulted in higher percentages of bifidobacteria after 26 wk of intervention (59.8% *vs* 47.2% in the scGOS/lcFOS group and the control group respectively). An increased microbial mass may partly explain the stool softening effect associated with infant milk formulas containing the specific scGOS/lcFOS (9:1) mixture compared to infant milk formulas lacking these oligosaccharides.

As mentioned before, selective fermentation of non-digestible components in the colon results in the production of SCFA. Infant milk formulas with scGOS/lcFOS (9:1) have indeed been shown to affect the production of SCFA, and to provide a faecal SCFA profile that is close to that of human milk fed infants, with higher percentages of acetate and lower percentages of propionate and butyrate[36,40]. In a study by Knol *et al*[40] the percentages of acetate, propionate and butyrate were 85.2%, 12% and 2.4% respectively in infants who received a formula with scGOS/lcFOS for 6 wk. In the control group, these percentages were 77.2%, 17.8% and 4% respectively after 6 wk of intervention. As SCFA can affect stool characteristics *via* stimulation of gastrointestinal motility by being used as energy source for colonic epithelial cells, or by inducing phasic and tonic contractions in circular muscle[21-25], scGOS/lcFOS (9:1) may positively stimulate the gastrointestinal motility.

NDO may also stimulate upper gastrointestinal motility. In a study bv Indrio *et al*[44] a beneficial effect of scGOS/lcFOS (9:1) on gastric emptying time was observed in preterm infants that received a formula with 0.8 g/L scGOS/lcFOS (9:1) or a control formula. In this study, it is suggested that the production of SCFA in the scGOS/lcFOS (9:1) group was responsible for an increased upper gastrointestinal motility, observed as a significant increased percentage propagation (assessed by electrogastrography) in the scGOS/lcFOS group when comped to the placebo group, and increased gastric emptying rate, observed as lower half emptying time in the scGOS/lcFOS group than in the placebo group[45]. As mentioned before, similar results have been observed in the study by Mihatsch *et al*[33] with a significantly shorter transit time (12 h) in preterm infants that had received a formula with scGOS/lcFOS, when compared to infants who had received a control formula (25.6 h) for 14 d. The gastrointestinal transit time measured in this study in the scGOS/lcFOS (9:1) group was mentioned to be within the normal range of what is observed human milk-fed infants (not further specified in the paper).

Both a direct effect as well as fermentation related effects can be affected by the type and structure of NDO[46]. The specific mixture of scGOS/lcFOS (9:1) consists of a combination of short-chain galacto-oligo-

saccharides and long-chain fructo-oligosaccharides in a ratio of 9:1. The composition and ratio of this specific mixture was specifically developed to closely resemble the molecular size composition of HMO (assessed by Mass Spectrometry)[26]. *In vitro* studies have indicated that short chain oligosaccharides are mainly fermented in the proximal part of the colon, while longer-chain NDO are mainly fermented in the distal colon[47]. Indeed, in a study by Moro *et al*[27] it was shown that part of the scGOS/lcFOS (9:1) is still detectable in faeces of infants, indicating that even in the distal part of the colon, not all scGOS/lcFOS (9:1) is fermented. This indicates that scGOS/lcFOS (9:1) can be effective throughout the whole gastrointestinal tract.

Although a direct correlation between stool consistency and/or stool frequency and bowel habit has never been shown, both stool frequency and stool consistency have often been used to assess bowel habit in a daily or clinical setting[48]. Recently, Russo *et al*[48] indicated that especially stool consistency is correlated with whole gut transit time, and that stool frequency is a poor marker for whole gut transit time. As described in the study of Mihatsch *et al*[33] with scGOS/lcFOS (9:1), gut transit time was assessed in preterm infants. In this study, gut transit time was significantly shorter in preterm infants receiving an infant milk formula with scGOS/lcFOS (9:1) when compared to an infant milk formula without scGOS/lcFOS (9:1). As an increased whole gut transit time can be a risk factor in the onset of constipation[49], scGOS/lcFOS (9:1) may have clinical relevance in the prevention of constipation by reducing whole gut transit time.

Effects of scGOS/lcFOS (9:1) on stool frequency were less often observed than effects on stool consistency. This can be due to the fact that the method of assessing stool frequency. The assessment of stool frequency was often done by the collection of faeces-containing diapers. However, it is difficult to assess whether a diaper contains one or more stooling episodes, and therefore, data collection may be less accurate. This may explain why effects on stool frequency are less commonly observed than effects on stool consistency. However, for these young infants there are no other methods available for a more accurate measurement of stool frequency.

CONCLUSION

In several of the studies with scGOS/lcFOS in a ratio of 9:1 in infant milk formulas, positive effects of this mixture on stool characteristics such as stool consistency and stool frequency were observed. Effects on stool consistency were more often found to be significant than the effects on stool frequency. As stool consistency has been shown to be correlated to whole gut transit time, stool softening effects of scGOS/lcFOS (9:1) may have a benefit in reducing the risk of constipation. This is crucial for the prevention of this very common functional gastrointestinal disorder in the general population, since one third of children followed up beyond the puberty continue to have severe complaints of constipation[50].

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REVIEW

Interventional treatment for unresectable hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of cancer-related death in the world. The Barcelona clinic liver cancer classification is the current standard classification system for the clinical management of patients with HCC and suggests that patients with intermediate-stage HCC benefit from transcatheter arterial chemoembolization (TACE). Interventional treatments such as TACE, balloon-occluded TACE, drug-eluting bead embolization, radioembolization, and combined therapies including TACE and radiofrequency ablation, continue to evolve, resulting in improved patient prognosis. However, patients with advanced-stage HCC typically receive only chemotherapy with sorafenib, a multi-kinase inhibitor, or palliative and conservative therapy. Most patients receive palliative or conservative therapy only, and approximately 50% of patients with HCC are candidates for systemic therapy. However, these patients require therapy that is more effective than sorafenib or conservative treatment. Several researchers try to perform more effective therapies, such as combined therapies (TACE with radiotherapy and sorafenib with TACE), modified TACE for HCC with arterioportal or arteriohepatic vein shunts, TACE based on hepatic hemodynamics, and isolated hepatic perfusion. This review summarizes the published data and data on important ongoing studies concerning interventional treatments for unresectable HCC and discusses the technical improvements in these interventions, particularly for advanced-stage HCC.

Key words: Unresectable; Hepatocellular carcinoma; Intermediate-stage; Advanced-stage; Interventional

Core tip: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. Interventional treatments for intermediate-stage HCC patients such as transcatheter arterial chemoembolization (TACE), drug-eluting bead embolization, and radioembolization, continue to evolve, improving prognosis. However, advanced-stage HCC is typically treated only with sorafenib, with only a modest improvement in overall survival. More effective therapies such as combined TACE and radiotherapy, TACE with special techniques, and isolated hepatic perfusion have been studied extensively. This review summarizes data on published and important ongoing studies concerning interventional treatments for unresectable HCC and discusses technical improvements in these interventions, particularly for advanced-stage HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths in the world[1]. The Barcelona clinic liver cancer (BCLC) classification is the current standard classification system for clinical management of patients with HCC (Table 1)[2] and suggests that patients presenting with very early-stage and early-stage disease (20%-30% of HCC patients) are suitable for curative treatments, such as resection, liver transplantation, local ablation with percutaneous ethanol injection, or radiofrequency ablation (RFA)[3,4]. Typically, patients with intermediate-stage disease show a survival benefit with transarterial chemoembolization (TACE). Patients with advanced-stage HCC with macroscopic vascular invasion (portal vein invasion), extrahepatic spread (lymph nodes and metastases), or cancer-related symptoms (performance status 1-2) may have a modest improvement in prognosis from first-line treatment with sorafenib, a molecularly targeted drug[5], while patients with terminal-stage disease (10%-20% of HCC patients) receive only symptomatic treatment.

TACE is the current standard of care for patients with intermediate-stage disease and has been reported to prolong survival and allow control of HCC symptoms[6-8]. Patients with advanced-stage HCC receive first-line therapy with sorafenib, according to the BCLC treatment algorithm, and an increase in overall survival and time to progression has been shown in 2 major trials[5,9]. However, the median overall survivals in patients treated with sorafenib for advanced-stage disease were 10.7 and 6.5 mo in the SHARP and Asia-Pacific trials, respectively, and a better treatment modality is needed.

The aims of this review are to summarize the published data and important ongoing studies concerning interventional managements for HCC and to discuss the technical improvements in TACE, particularly for advanced-stage HCC.

UNRESECTABLE HCC

Intermediate-stage HCC

Patients presenting with Child-Pugh class A and B liver function and large or multinodular HCC without cancer-related symptoms, macrovascular invasion, or extrahepatic metastasis are diagnosed with intermediate-stage HCC. Transarterial intervention, particularly TACE, is the recommended BCLC classification treatment and is endorsed by the European Association for the Study of the Liver[10] and the American Association for the Study of Liver Diseases[11] guidelines.

TACE: The liver is a unique organ with a dual blood supply provided by the portal vein and hepatic artery. The portal vein delivers 80% of the blood supply to healthy liver tissue, while, the hepatic artery delivers 99% of the blood supply to hepatic tumors. Based on this observation, TACE for HCC is appropriate for patients for whom surgical or percutaneous ablative treatment is contraindicated. TACE involves the injection of anticancer drugs (doxorubicin, epirubicin, cisplatin or miriplatin) and iodized oil (Lipiodol Ultra Fluide, Laboratoires Guerber, Aulnay-sous-Bois, France) into the hepatic artery, followed by the administration of embolic agents[12,13]. The antitumor effect of TACE is greater than that of either anticancer drugs[14] or iodized oil[15,16] alone. Prognostic factors found to be associated with that a reduction in serum alpha fetoprotein levels after the intervention[17], the number of TACE procedures[18,19], a low model for end-stage liver disease score[20], the absence of diffuse disease[21,22], and small tumor size[23] have been shown to correlate with better survival.

Table 1 Barcelona-clinic liver cancer classification in patients diagnosed with hepatocellular carcinoma

Stage	Description
Very early	PS 0, Child-Pugh A, single HCC < 2 cm
Early	PS 0, Child-Pugh A-B, single HCC or 3 nodules < 3 cm
Intermediate	PS 0, Child-Pugh A-B, multinodular HCC
Advanced	PS 1-2, Child-Pugh A-B, portal neoplastic invasion, nodal metastases, distant metastases
End-stage	PS > 2, Child-Pugh C

This classification is in accordance with that outlined in reference [2]. PS: Performance status; HCC: Hepatocellular carcinoma.

Iodized oil is used as an embolic agent and carrier of anticancer drugs in TACE. Mixtures of anticancer drugs and iodized oil are classified as either emulsions (oil with saline and drugs) or suspensions (drugs in oil)[24,25]. Comparative studies of suspensions versus emulsions in TACE, with cisplatin powder[26] or epirubicin[27] as anticancer agents for the treatment of rabbit VX2 liver tumors, demonstrated that suspensions were superior to emulsions with regard to their antitumor effects. Several factors are thought to explain these findings. In an emulsion, most of the powdered drug is contained in the unstable aqueous phase[13,28], which undergoes rapid dilution in the blood, elimination from the hepatic tissue, and excretion by the kidneys. In a suspension, the powder is directly mixed in the oil phase and distributed in a similar fashion to iodized oil alone through the portal venules and sinusoids over a 24-h period[29,30]. As a result, suspensions show a longer anticancer drug release time at the tumor border and higher continuous drug concentrations. This longer tissue/drug activity period associated with the use of suspensions produces superior antitumor effects, as evaluated by changes to the growth ratio and results of histopathological investigations[24,25,31,32]. However, since most anticancer drugs are hydrophilic, it is hard to make stable drug suspensions in oil. Additionally, tumor uptake of suspensions is poor, most likely due to their high viscosity[33]. As

the viscosity of iodized oil has a negative correlation with temperature exceeding 50 mPa.s at 20° but decreasing to 22 mPa.s and 12 mPa.s, at 40° and 60°, respectively[34], we designed a syringe warmer to maintain suspensions at a high temperature. We have obtained significant antitumor effects without major adverse events when treating HCC patients with warmed drug suspensions since January of 2011 (article submitted for publication), and a prospective, comparative study of TACE with warmed suspension at 53° is currently under way.

Drug-eluting beads (DEB), which can be loaded with doxorubicin, represent a novel drug delivery system for chemoembolization. A slow and sustained release of drug is predicted after intra-arterial delivery of DEB, and several studies have reported this method to be safe and efficacious in the treatment of HCC[35,36]. DEB-TACE is proposed to have a better pharmacokinetic profile than conventional Lipiodol-TACE and result in decreased systemic adverse effects[37]. However, a recent study reported that liver/biliary injury was strongly associated with DEB-TACE irrespectively of the type of tumor and underlying liver disease. At least 1 liver/biliary injury was observed after 30.4%-35.7% of DEB-TACE sessions, whereas this event occurred after only 4.2%-7.2% of conventional Lipiodol-TACE ($P < 0.001$)[38]. Despite encouraging pharmacological and antitumor advantages, a recent large, phase Ⅱ randomized trial of HCC failed to demonstrate a better tumor response rate to DEB-TACE compared with conventional Lipiodol-TACE[39]. Further randomized, controlled, multicenter trials are necessary to explore the differences in adverse events and to assess the effects of DEB-TACE on short- and long-term outcomes.

Radioembolization involves the catheter-based delivery of yttrium-90 (^{90}Y)-embedded resin (SIR-Spheres ®; Sirtex Medical, Sidney, New South Wales, Australia) or glass (TheraSphere®, MDS Nordion, Toronto, Ontario, Canada) microspheres into the hepatic artery[40] to distribute radioisotopes to the tumor. ^{90}Y is a pure beta-emitter, which generates high-energy radiation with a short half-life (2.67 d) and tissue penetration (mean 2.5 mm, maximum 11 mm)[41]. Once administered, these microspheres selectively emit high-energy, low-penetration radiation to the tumor, resulting in necrosis. Although there is currently a lack of sufficient prospective data comparing treatment efficacy in terms of response or survival between radioembolization and TACE for intermediate-stage HCC, a retrospective study[42] comparing the outcomes between radioembolization ($n = 38$) and TACE ($n = 35$) determined no significant difference in survival (median 8.0 mo *vs* 10.3 mo, $P = 0.33$). However, postembolization syndrome was significantly more severe in patients who underwent TACE[42]. Further evaluation of radioembolization, including direct comparisons with TACE, is needed.

Radioembolization may cause injury to the hepatic tissue and result in fibrotic changes and tissue atrophy[43,44]. The loss of liver function can be compensated for by non-treated liver segments, which tend to undergo hypertrophy after radioembolization (the atrophy-hypertrophy complex)[45]. A few studies have described the hepatic volume changes of treated and non-treated liver segments after radioembolization and report a 40%-55% loss in volume in treated segments and a 30%-40% increase in volume in non-treated segments[46,47].

Combination TACE and RFA: RFA is a curative treatment for very early- and early-stage HCC but is limited in the control of large tumors. The effectiveness of RFA is dependent on thermal necrosis, and blood flow through the tumor promotes heat loss and prevents proper heating. TACE can be performed strategically before RFA treatment to reduce this heat sink effect and increase the ablation volume of the tumor. A recently published, randomized study[48] showed that, when patients with tumors > 3 cm were randomized to receive either TACE, RFA, or TACE-RFA, the combination of TACE-RFA produced a superior median survival (TACE-RFA: 37 mo, TACE: 24 mo, and RFA: 22 mo) and rate of objective tumor response (TACE-RFA: 54%, TACE: 35%, and RFA: 36%). The results of TACE-RFA support the combining of locoregional modalities to improve the outcomes of patients with unresectable HCCs.

Modified TACE to obtain complete necrosis of the tumor: Histopathological investigations of HCCs resected after TACE have shown that the most viable tissue is located at the periphery of the tumor[49]. The efficacy of TACE is limited by the dual blood supply of liver tumors, which makes it impossible to deliver anticancer agents to the entire tumor area or achieve sufficient tumor ischemia without irreversible damage to surrounding normal liver parenchyma. Some studies[50,51] have reported superselective TACE to be useful for the treatment of small HCCs because it can embolize both the tumor and surrounding normal parenchyma. For large liver tumors, however, the therapeutic options are limited to techniques that result in complete necrosis of the tumor.

To obtain complete necrosis of a HCC, including the periphery, several reports[52-54] have investigated the hemodynamic changes occurring in the liver and tumors during hepatic vein balloon occlusion using computed tomography during hepatic arteriography (CTHA) and arterial portography, demonstrated that the occluded area is supplied with arterial blood alone[53,54], and suggested that sufficient embolization may be obtained during TACE with arterial control alone. Since balloon occlusion of the segmental hepatic vein eliminates the dual blood supply, leaving only the arterial supply, TACE performed using this technique can sufficiently embolize both the tumor and the surrounding liver parenchyma. We have performed TACE under balloon occlusion of the hepatic vein for more than 70 patients with advanced HCC, but only 30% of the patients benefited from the procedure due to the complex veno-venous communications in the liver[34,53]. The hepatic veins assume the role of draining

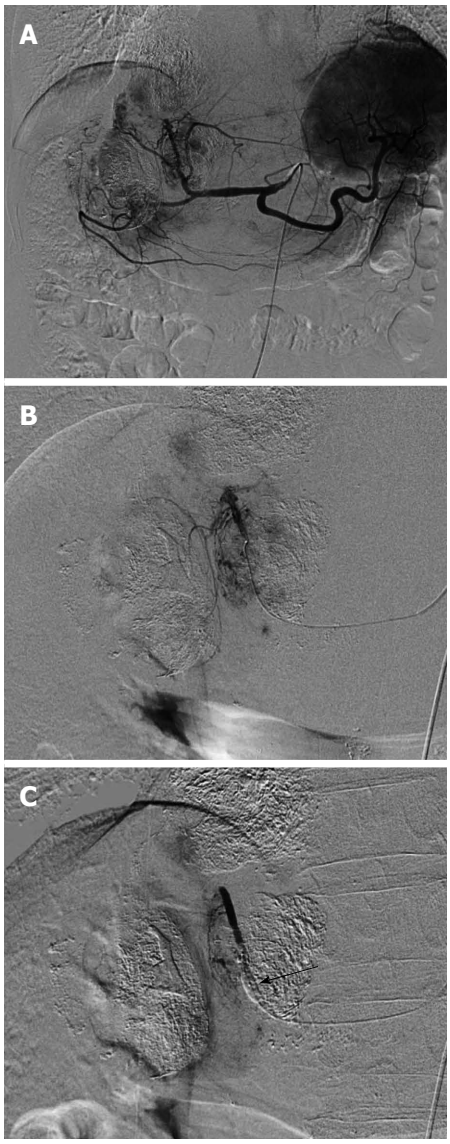

Figure 1 Selective arteriography with and without balloon-occluded feeding artery in a 72 year-old man with multiple hepatocellular carcinomas. A: Celiac arteriography revealed multiple hepatocellular carcinomas (HCCs) in the liver; B: Opacified HCCs were shown by medial segmental arteriography without balloon-occluded feeding artery; C: In contrast, the arteriography with balloon occlusion showed faint opacification of HCCs and nontumoral liver parenchyma was opacified. Arrow indicated an inflated microballoon.

veins despite single hepatic vein occlusion[53,55]; therefore, sufficient embolization of large liver tumors using the hepatic vein balloon occlusion technique is difficult.

When portal venous blood flow is decreased or stopped due to tumor thrombus, thromboembolus, or compression of the portal vein, the affected liver parenchyma appears as a hyperattenuated area with straight borders on contrast CT[56-62]. This appearance is similar to that seen with hepatic vein occlusion. One study[63] investigated the hepatic hemodynamic changes under acute balloon occlusion of the portal vein using single-level dynamic CTHA and reported several phenomena. First, a demarcated, hyperattenuated area of the liver parenchyma was noted in the distribution of the occluded portal vein branch, which was significantly higher than that of the non-occluded area ($P < 0.01$). Second, the balloon-occluded portal branch enhancement appeared to result from arterioportal communications. These findings suggest that, when portal venous flow stoppage occurs, only the hepatic artery supplies the corresponding area and hepatic arterial blood flow is increased. This phenomenon is known as the hepatic artery buffer response. The observed variations in blood flow are due to the degree of adenosine clearance, to which the hepatic artery is very sensitive, as adenosine is a potent vasodilator of the hepatic artery[64-66]. Finally, there is little anatomical variation in portal veins or porto-portal venous anastomosis. Therefore, sufficient embolization may occur, even in large liver tumors, by TACE under the corresponding portal vein branch occlusion (TACE-PVO). We began using TACE-PVO for large HCCs 6 years ago and have seen promising antitumor effects, which will be reported in the near future.

In 1981, Yamada *et al*[67] first reported a balloon-occluded arterial infusion therapy for liver tumors. In the 1990s, compulsory, superselective, balloon-occluded arterial embolization using a 3-French microballoon catheter[68] was introduced for hypovascular liver tumors. The selective balloon-occluded TACE (B-TACE) method is currently a useful treatment for HCC because of preventing proximal migration and leakage of embolization materials[69]. This technique leads to dense lipiodol emulsion accumulation in the HCC nodule[69]; however, the effectiveness of this therapy for HCC is controversial.

CT arteriography under balloon occlusion of the sub- or segmental hepatic artery demonstrates a much higher attenuation in the corresponding liver parenchyma than in HCC tumors[70]. This phenomenon suggests that B-TACE leads to dense lipiodol emulsion accumulation in nontumorous liver parenchyma and not in HCC nodules. Indeed, arteriography *via* the balloon-occluded hepatic artery shows dense opacification in the liver parenchyma and faint opacification in the HCC, while arteriography *via* the non-occluded hepatic artery shows dense opacification in the HCC (Figure 1). Therefore, the effectiveness of B-TACE is controversial. To remedy this, we developed a two-step B-TACE method for liver tumor (Figure 2). First, tumors are sufficiently embolized by TACE, followed by the inflation of a microballoon catheter to occlude the feeding artery in the second step. B-TACE is then performed for full embolization of the peritumoral liver parenchyma. Following this two-step B-TACE procedure, CT images showed dense lipiodol accumulation in the HCC. The two-step B-TACE method has a great advantage for therapy in patients with liver dysfunction because it prevents proximal migration and leakage of embolization materials, the embolized area can be determined, and injury to the liver parenchyma can be prevented. A comparative study of the two-step B-TACE method with conventional TACE obtained promising results for two-step B-TACE.

Advanced-stage HCC

HCC patients with Child-Pugh class A and B liver func-

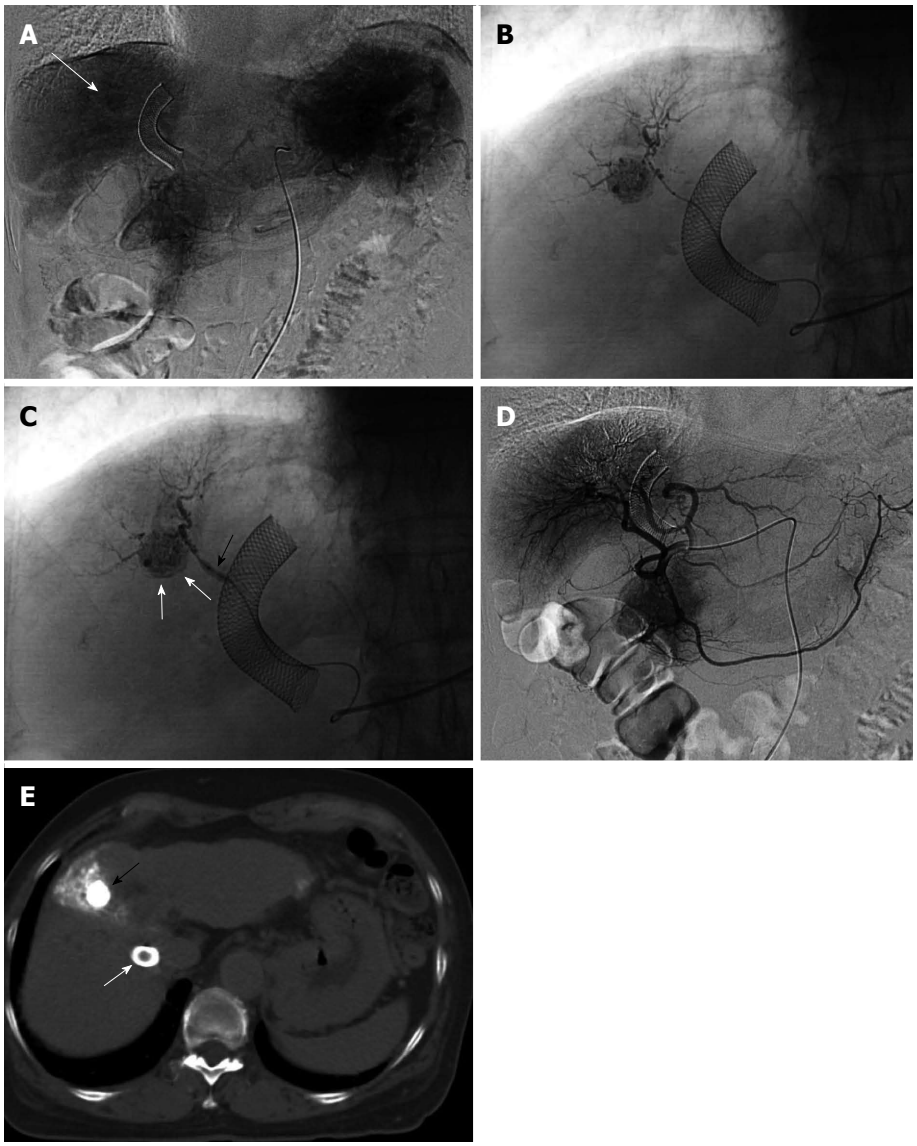

Figure 2 Two-step balloon-occluded transarterial chemoembolization. A 76-year old woman characterized as Child-Pugh class C underwent transjugular intrahepatic portosystemic shunt (TIPS) for massive ascites before several transarterial chemoembolization (TACE) sessions. Celiac arteriography in the venous phase (A) showed a small hepatocellular carcinoma (HCC) (arrow). A microballoon catheter was advanced into the tumor feeding artery. Then, two-step balloon-TACE (B-TACE) was performed. First, tumors were sufficiently embolized by TACE without balloon occlusion of the tumor feeding artery (B); Second, a microballoon catheter was inflated to occlude the feeding artery (C, black arrow), and B-TACE was performed to fully embolize the peritumoral liver parenchyma (C, white arrows). Common hepatic arteriography showed no proximal migration or leakage of embolization materials (D). Following the two-step B-TACE procedure, computed tomography showed dense lipiodol accumulation in the HCC (E, black arrow). There was no evidence of proximal migration or leakage of embolization materials. White arrows indicated a stent for TIPS.

tion who present with macroscopic vascular invasion, extrahepatic spread, or cancer-related symptoms (performance status 1-2) are diagnosed with advanced-stage HCC. First-line treatment with sorafenib is recommended for this stage of HCC according to the BCLC treatment algorithm based on the results of sorafenib therapy in randomized trials in Europe and Asia[5,9]. However, overall survival was only modestly improved by 2.8 mo[5]. Patients with advanced-stage HCC require more effective therapy than sorafenib or conservative treatment, and this is currently under consideration.

HCC with vascular invasion: In general, HCCs with portal venous tumor thrombus (PVTT) and significant arterioportal or arteriohepatic vein shunts are contraindicated for TACE. Conventional TACE did not provide a survival benefit for advanced-stage patients with PVTT[71], potentially due to hepatic infarction and acute liver failure. Portal vein invasion is a major prognostic factor for reduced survival in patients with advanced HCC. Advanced HCC with PVTT in the major branches of the portal vein, particularly the main portal vein, has an extremely poor prognosis (3-4 mo without treatment[72]) because portal vein invasion may cause liver failure, massive esophageal variceal bleeding, or extensive tumor spreading throughout the liver[73]. The results of sorafenib therapy in randomized trials in Europe and Asia[5,9] did not provide a substantial survival improve-

ment in patients with PVTT. As the incidence of patients with HCC and PVTT was reported to be between 12.5% and 39.7% at the time of diagnosis using recent imaging techniques[74], effective therapies are needed for advanced HCC patients with vascular invasion.

Combined TACE and radiotherapy (TACE + RT) consists of TACE followed by radiation therapy to the vascular invasions and has shown an overall survival benefit in patients with PVTT[75-77]. Shirai *et al*[75] reported that HCC patients (n = 19) with PVTT ⩾ 8 cm in the first branch or main trunk that received TACE + RT had 1- and 2-year survival rates of 47.4% and 23.7%, respectively. Although prospective data comparing the efficacy of TACE + RT and sorafenib therapy in terms of response or survival in advanced-stage HCC with PVTT are not sufficient, a retrospective study[78] comparing the outcomes of TACE + RT (n = 27) and sorafenib therapy (n = 27) in a propensity score-matched cohort determined that the TACE + RT group had prolonged overall survival compared to the sorafenib group (6.7 mo *vs* 3.1 mo, $P < 0.001$). To determine the efficacy of TACE + RT compared to sorafenib in locally advanced HCC, a randomized controlled multicenter trial is necessary.

HCC has a tendency to spread to the portal vein and, to a lesser extent, the hepatic vein[79], which allows the development of arteriovenous shunts. These shunts represent the main impediment to successful TACE because anticancer drugs or mixtures of iodized oil and anticancer drugs easily pass through them[80]. Conventional TACE is therefore not effective for HCC patients with hepatic arteriovenous shunts and may even be harmful, due to the possibility of pulmonary embolism[81-83]. These patients require an effective, low-risk alternative treatment option.

RFA may be a useful treatment for HCCs < 3 cm in diameter; however, most HCCs with intratumoral arteriohepatic vein shunts are large, and RFA cannot be performed. To overcome this limitation, we performed TACE of the HCC feeding arteries with balloon occlusion of the corresponding draining hepatic vein, which was monitored by angiography and CT[34]. If the target HCC is located in the lateral segment of the liver, we recommend TACE with balloon occlusion of the left hepatic vein. If the target HCC is located in the right or middle lobe of the liver, particularly the upper portion, we recommend TACE with balloon occlusion of 2 hepatic veins, typically the right and middle hepatic veins[55]. TACE with balloon occlusion of the corresponding hepatic vein results in both significant tumor growth control and elimination of the intratumoral shunts[34]. After this modified procedure, conventional TACE can be performed for the treatment of residual HCC.

As noted above, HCC is frequently associated with arteriovenous shunts, which typically present as arterioportal shunts. Kojiro[84] analyzed 106 resected HCCs < 2 cm in diameter and found that nodular-type HCC was associated with microscopic portal invasion in up to 25% of cases. Although the presence of small arterioportal shunts does not necessarily preclude TACE therapy for unresectable HCC, larger arterioportal shunts caused by tumor invasion interfere with TACE because anticancer drugs, either alone or mixed with iodized oil, easily pass through the shunts[81,82]. Conventional TACE causes extensive embolization of the portal vein and can induce extensive ischemia of nontumorous liver parenchyma[80]. The presence of significant arterioportal shunts can also lead to liver dysfunction and portal hypertension, resulting in potentially life-threatening conditions such as rupture of the gastroesophageal varices[85-88], refractory ascites, or hepatic encephalopathy.

Several studies have treated significant arterioportal shunts by embolization of the hepatic arteries with materials such as gelatin sponges or coils, and these approaches have yielded good short-term results[89,90]. However, they do not eradicate the HCC and contribute little to patient survival[89,90]. We attempted to overcome this complication by performing TACE of tumor-feeding arteries with occlusion of the corresponding portal vein[91]. The intrahepatic portal branch was punctured under ultrasonographic guidance using an 18-gauge percutaneous transhepatic cholangiography needle. For TACE-PVO, a 5- or 8- French balloon catheter was advanced into the portal vein branch identified by direct portography and hepatic arteriography to contain the arterioportal shunt. The balloon catheter was inflated, and a mixture of lipiodol (up to 15 mL) and anticancer agents, as an emulsion or suspension, was injected *via* the target feeder artery until reflux into the hepatic artery was confirmed. Particles of gelatin sponge were immediately injected into the feeder artery until the target hepatic artery was occluded. If the target HCC concurrently invades the right and left branches of the portal vein, we recommend TACE with balloon occlusion of the 2 portal vein branches (Figure 3).

The effectiveness of TACE-PVO for arterioportal shunts was ascribed to balloon occlusion of the corresponding portal veins, resulting in adequate embolization of the entire tumor, including the portions involving arterioportal shunts. Consequently, TACE-PVO may prevent the development of collateral anastomoses to arterioportal shunts.

We performed a comparative, prospective, but not randomized study of standard transarterial embolization (TAE) or TACE versus TACE-PVO for HCC with significant arterioportal shunts[91]. Subjects differed fundamentally by patients' choice of treatment. We found that TACE-PVO was significantly better (P = 0.009) than standard TAE for arterioportal shunt treatment, and subsequent angiographic findings suggested the superiority of TACE-PVO (P = 0.028). Antitumor response (P = 0.002) and patient outcome (P = 0.032) were significantly better in the TACE-PVO group than in the standard treatment group. Furuse *et al*[90] reported that HCC patients with significant arterioportal shunts due to PVTT who underwent embolization had 1- and 2-year survival rates of 12% and 0%, respectively. In our study, 1- and 2-year survival rates in the standard treatment group were 28.6% and 0%, respectively, similar to that observed by

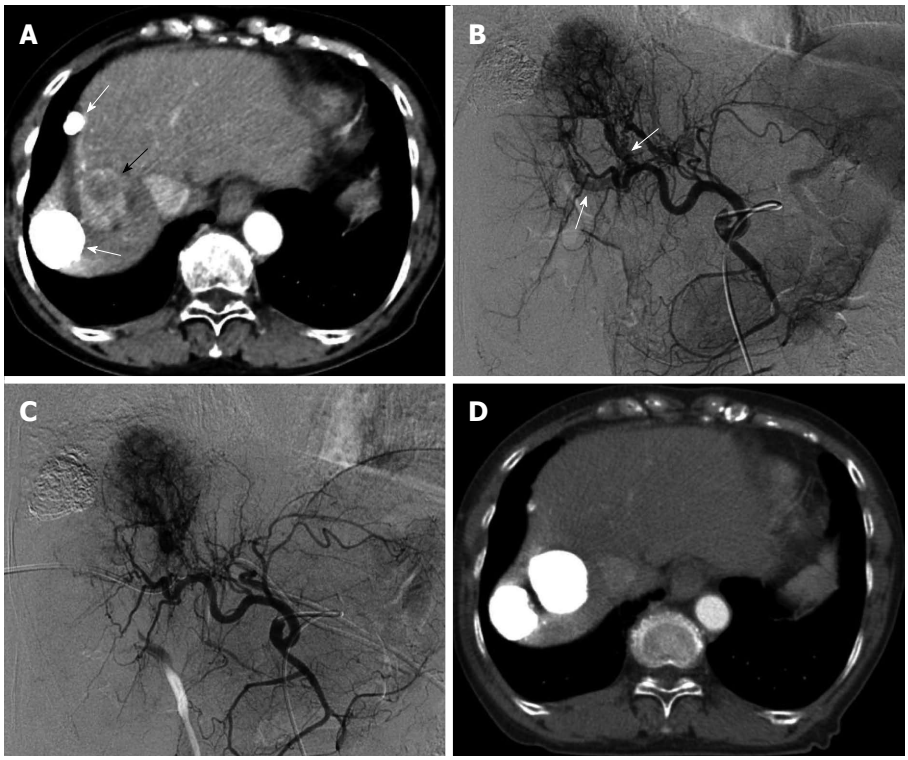

Figure 3 Multiple hepatocellular carcinomas with significant arterioportal shunts in a 71-year-old woman. Enhanced computed tomography (CT) imaging revealed viable hepatocellular carcinomas (HCCs) (A, black arrow) with lipiodol deposit HCCs (A, white arrows). Common hepatic arteriography in the arterial phase (B) demonstrated HCC with significant arterioportal shunts (B, arrows), consisting of P8 and P4. Common hepatic arteriography under balloon occlusion of the portal vein branches of P8 and P4 showed an opacified tumor with non-visualized arterioportal shunts (C). CT images 2 mo after transcatheter arterial chemoembolization under occlusion of the 2 portal vein branches (D) demonstrated a dense lipiodol deposit in the HCC and a complete response, according to modified Response Evaluation Criteria in Solid Tumors.

Furuse *et al*[90]. We obtained both a good target tumor response and dramatic improvement in arterioportal shunts with TACE-PVO therapy, with favorable 1-, 2-, and 3-year survival rates of 85.7%, 64.3%, and 42.9%, respectively[91]. This survival was markedly better than that in the previous series.

To our knowledge, no effective treatment for both HCC and significant arterioportal shunts has been previously reported. We have performed TACE-PVO on 37 patients with significant arterioportal shunts at our institution, resulting in good tumor responses and prolonged survival. Randomized controlled multicenter trials are necessary to further explore differences in quality of life and to assess the effects of TACE-PVO on short- and long-term outcomes.

Finally, we introduce a specialized embolization technique for HCC with arterioportal shunt. A patient presented to our institution with multiple HCCs and underwent several sessions of TACE. Following therapy, 1 HCC, fed by a cystic artery with an arterioportal shunt, remained. We attempted superselective TACE-PVO *via* the branch of the cystic artery, thus avoiding embolization; however, the procedure was not successful (Figure 4). We then performed chemoembolization *via* the portal vein branch with an arterioportal shunt during balloon occlusion of the portal vein (TPCE) and observed a dense accumulation of lipiodol in the tumor. Angiography 3 mo after the TPCE procedure revealed no arterioportal shunt, and conventional TACE was performed for the residual HCC. To our knowledge, this is the first report of successful TPCE, and this technique may be a useful and safe method for selected patients with HCC and arterioportal shunts.

Isolated hepatic perfusion (IHP) is an attractive option for achieving higher tumor exposure to any given agent while avoiding systemic toxicity by decreasing systemic drug exposure. Surgical IHP has shown promising results[92-94]; however, this technique is therapeutically limited because it requires an aggressive surgical intervention that is associated with considerable morbidity and mortality. Further, this technique is not amenable to repeated interventions, mainly due to the formation of adhesions[92,94,95]. Therefore, several studies have attempted to develop safe and repeatable percutaneous IHP techniques without using laparotomy with balloon occlusion catheters. Ku *et al*[96] reported on advanced-stage HCC patients (n = 25) that underwent percutaneous IHP after reductive surgery and determined their 5-year overall survival rate to be 42%. However, percutaneous IHP techniques result in higher rates of leakage from the perfusion circuit into the systemic circulation[97-99], mainly because the distance between the right atrium and hepatic vein origins is often too short to allow balloon occlusion of the suprahepatic inferior vena cava (IVC) above the hepatic veins without

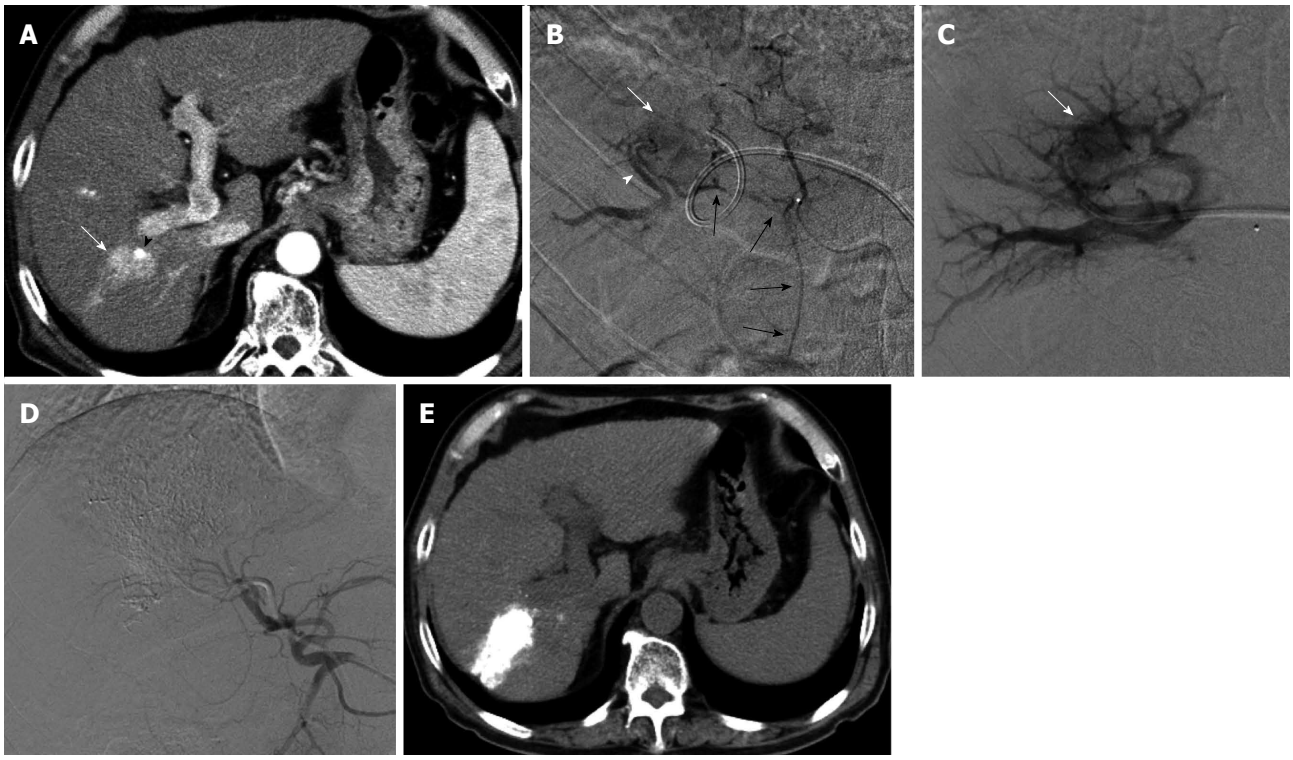

Figure 4 Transportal chemoembolization for hepatocellular carcinoma with an arterioportal shunt in a 70-year-old man. Enhanced abdominal computed tomography (CT) examination (A) demonstrated a recurrent hepatocellular carcinoma (HCC) (A, arrow) in the posterior segment with a lipiodol deposit (arrowhead). Hepatic arteriography *via* the anterior branch (B) revealed a HCC (B, white arrow) fed by the cystic artery (B, black arrows) and an intratumoral arterioportal shunt (B, arrowhead). A 5-French sheath was inserted into the portal vein *via* the lateral superior branch of the left portal vein. Direct portography *via* the portal vein branch contributed an arterioportal shunt showed a tumor stain (C, arrow). Following transcatheter portal chemoembolization, proper hepatic arteriography showed non-visualized tumor stain and arterioportal shunt (D). Non-enhanced CT 7 d after therapy (E) showed a dense accumulation of lipiodol in the tumor and peritumoral liver parenchyma.

occluding the hepatic veins themselves[97,99]. IHP involves an orthograde approach, employing inflow *via* the hepatic artery and outflow *via* the IVC through the hepatic veins. All non-surgical orthograde IHP techniques may be associated with this problem, including combinations of orthograde percutaneous and surgical IHP techniques that employ a double-balloon catheter for IVC occlusion.

To overcome this disadvantage, we developed a percutaneous IHP circulation system - retrograde-outflow percutaneous IHP (R-PIHP) - by redirecting hepatic outflow through the portal vein[100,101] based on hepatic circulation under hepatic vein occlusion[53,54]. This technique inhibits systemic leakage and maintains a high concentration of drug delivery to the liver. A phase Ⅰ/Ⅱ trial of R-PIHP for unresectable liver malignancies, including HCC, was begun 3 years ago, and we have obtained good tumor responses and survival benefits although R-PIHP recommends expert interventional techniques (Figure 5).

Unresectable HCC usually presents as a hypervascular tumor. TACE results in both tumor hypoxia and longer activity periods for anticancer drugs remaining in the tumor tissues. However, TACE also induces a post-treatment surge of angiogenic factors, such as vascular endothelial growth factor (VEGF), which can occur as early as a few hours post-TACE[102]. This process may contribute to the revascularization of the tumor, thus reducing the efficacy of TACE[103,104]. Sorafenib is an antiangiogenic drug that blocks tumor cell proliferation and angiogenesis by inhibiting the activity of VEGF receptors. Combining sorafenib with TACE may potentially improve the treatment outcome[105]. Several studies have evaluated the efficacy and safety of this combination treatment[106,107], and clinical trials investigating this therapeutic approach are currently ongoing. The dosing schedule of antiangiogenic drugs in relation to TACE is a key factor for this method of therapy. A randomized phase Ⅲ study comparing the advent of sorafenib treatment with placebo 1-3 mo after TACE failed to show a survival benefit[106]. In contrast, a single-arm phase Ⅱ study with sorafenib beginning 1 wk after TACE reported a disease control rate of 95%[107], according to the response evaluation criteria for solid tumors. Optimal scheduling of antiangiogenic agents with TACE is essential to improve the patient prognosis. Moreover, if locoregional treatments are superior to conventional TACE, the combination of sorafenib with these other interventional treatments, such as DEB-TACE or radioembolization, is also promising.

CONCLUSION

In this review, we have summarized the efforts of many studies to improve the treatment outcomes of patients with intermediate- or advanced-stage HCC. For intermediate-stage HCC patients, interventional treatments, such

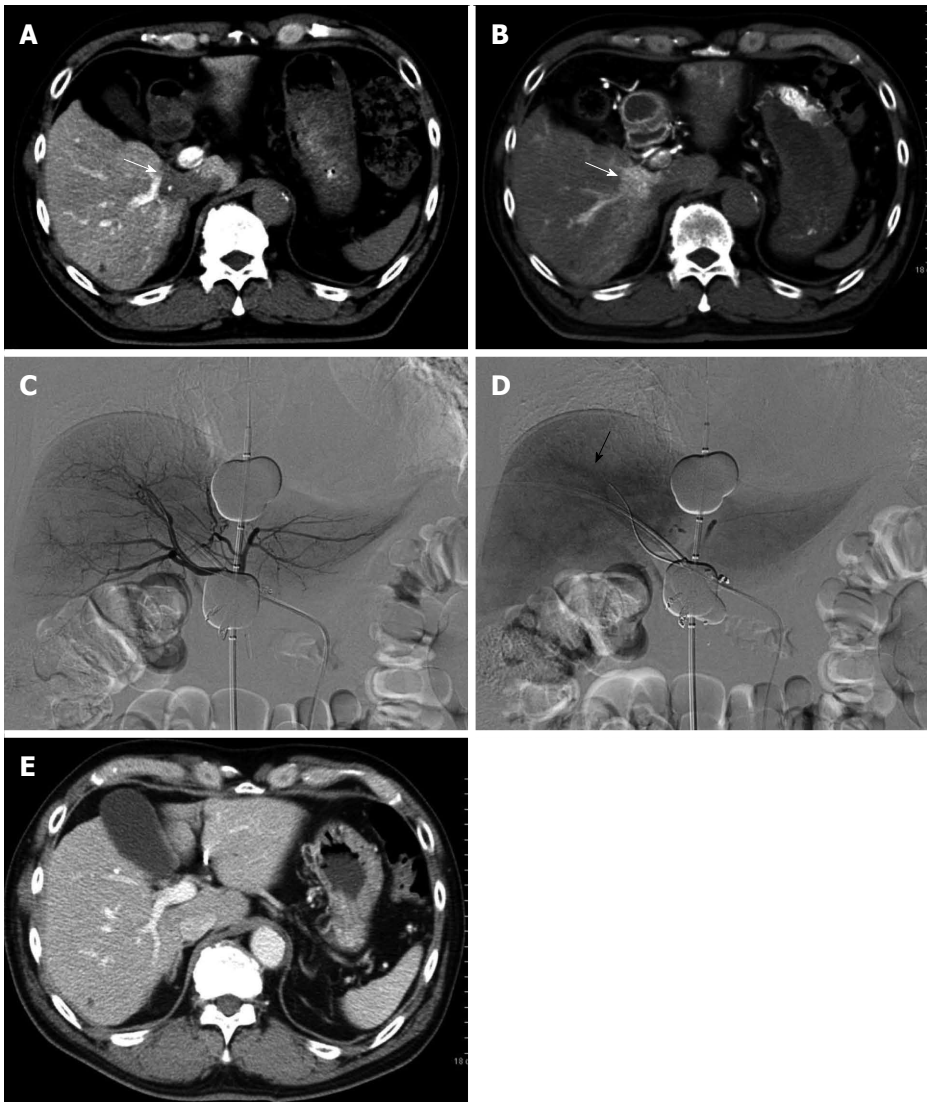

Figure 5 Retrograde-outflow percutaneous isolated hepatic perfusion for multiple hepatocellular carcinomas in a 64-year-old man. A patients received 2 sessions of transcatheter arterial chemoembolization for multiple hepatocellular carcinomas (HCCs) at other hospital. Computed tomography (CT) during arterial portography (A) and hepatic arteriography (B) demonstrated multiple HCCs with portal vein invasion (arrows). Retrograde-outflow percutaneous isolated hepatic perfusion (C, arterial phase; D, venous phase) was performed twice (arrow of D indicated a portal branch), and enhanced CT imaging 6 mo after therapy (E) revealed that the HCC was diminished.

as TACE, drug-eluting beads embolization, radioembolization, and combination therapies, for prognostic improvement are continually evolving. For advanced-stage HCC patients, sorafenib chemotherapy, TACE-based therapies combined with sorafenib treatment, TACE with radiotherapy, TACE based on hepatic hemodynamics, and IHP are all effective therapies. In conclusion, the evolution of therapy continues to improve the prognosis of patients with unresectable HCC.

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REVIEW

Predictive proteomic biomarkers for inflammatory bowel disease-associated cancer: Where are we now in the era of the next generation proteomics?

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Abstract

Recent advances in genomic medicine have opened up the possibility of tailored medicine that may eventually replace traditional "one-size-fits all" approaches to the treatment of inflammatory bowel disease (IBD). In addition to exploring the interactions between hosts and microbes, referred to as the microbiome, a variety of strategies that can be tailored to an individual in the coming era of personalized medicine in the treatment of IBD are being investigated. These include prompt genomic screening of patients at risk of developing IBD, the utility of molecular discrimination of IBD subtypes among patients diagnosed with IBD, and the discovery of proteome biomarkers to diagnose or predict cancer risks. Host genetic factors influence the etiology of IBD, as do microbial ecosystems in the human bowel, which are not uniform, but instead represent many different microhabitats that can be influenced by diet and might affect processes essential to bowel metabolism. Further advances in basic research regarding intestinal inflammation may reveal new insights into the role of inflammatory mediators, referred to as the inflammasome, and the macromolecular complex of metabolites formed by intestinal bacteria. Collectively, knowledge of the inflammasome and metagenomics will lead to the development of biomarkers for IBD that target specific pathogenic mechanisms involved in the spontaneous progress of IBD. In this review article, our recent results regarding the discovery of potential proteomic biomarkers using a label-free quantification technique are introduced and on-going projects contributing to either the discrimination of IBD subtypes or to the prediction of cancer risks are accompanied by updated information from IBD biomarker research.

Key words: Inflammatory bowel disease; Biomarker; Proteomics; Tailored medicine; Colitic cancer

Core tip: Our recent achievements in discovering biomarkers to predict cancer risk are introduced. Ultimately, models based on combinations of genotype and gene expression data referenced with clinical, biochemical, and serological data may permit the development of tools for individualized risk stratification and efficient treatment selection, as well as complete rescue from complications, including colitis-associated cancer, in the near future.

Park JM, Han NY, Han YM, Chung MK, Lee HK, Ko KH, Kim EH, Hahm KB. Predictive proteomic biomarkers for inflammatory bowel disease-associated cancer: Where are we now in the era of the next generation proteomics? *World J Gastroenterol* 2014; 20(37): 13466-13476 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13466.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13466

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that causes injury to the gastrointestinal (GI) tract and is accompanied by clinical characteristics of remission and relapse. The two common types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Although many molecular methods for the investigation of protein and gene sequences have contributed to diagnostic methodologies, the diagnosis of IBD is primarily based on clinical, endoscopic, radiological, and histological criteria. Unfortunately, there has been little to no change in this traditional approach to diagnosis, despite modern advances in genomics and proteomics. However, progress in treatment strategies involving the incorporation of marketed biologicals and molecular targeted therapeutics has lead to the development of the concept of "deep remission" or "mucosal healing" in the treatment of IBD[1,2]. Furthermore, there has been major innovation in diagnostic methods, including the development of more complicated endoscopic and non-invasive imaging methods. These techniques are used to improve quality of life (QOL) of patients, predict complications, and contribute to the prevention or surveillance of cancer associated with IBD such as colitis-associated cancer (CAC)[3].

Recent developments in the molecular pathogenesis of IBD have highlighted three aspects. First, IBD is caused by complex disorders influenced by susceptibility genes, and is characterized by disturbed epithelial barrier function, and abnormal innate and adaptive immunity. Second, the compositions of gut microflora are altered or the epithelial barrier function is disorganized, which leads to a response from the immune system. Third, a murine model has been very helpful in unraveling the pathogenesis/mucosal immunopathology of IBD[4] by suggesting that the abnormal immune reaction to normal microbiota results from dysregulation of the mucosal immune system[5]. For example, the composition of microbes in the gastrointestinal tract may impair the patient's lifestyle in developed countries and a pathogenic infection in the gastrointestinal tract has a significant function in modulating the immune system. These data may explain why developing and some Asian countries are confronting steep increases in the incidence of IBD. Developments in gene-sequencing technologies, such as next generation sequencing, as well as the emergence of several bioinformatic tools have led to novel insights into the microbe balance in the human gastrointestinal tract and the effect of microbes on human physiology and pathology[6].

Additional innovative technologies, such as mass spectrometry (MS)-based proteomics, also referred to as next generation proteomics, have discovered new classes of proteomic biomarkers that can be used to explore the accurate and comprehensive molecular characterization of IBD genes and proteomes. These advances are expected to lead to more reliable identification of IBD diagnostic- or progression-specific targets and enable molecular diagnosis, as well as provide guidance regarding the selection of treatment options and the risk of cancer development in cases with longstanding remission and relapse[7]. A more robust molecular definition of IBD subtypes is likely to be based on specific molecular pathways that determine not only disease susceptibility, but also disease characteristics, such as location, natural history and therapeutic response. Furthermore, such advances could be applicable in defining "deep remission", which has not been feasible with currently-used scoring systems or endoscopic evaluation. Discovering biomarkers for IBD may allow objective measurements of disease activity and severity while also serving as prognostic indicators for therapeutic outcomes[8]. Furthermore, the discovery of one or more biomarkers predictive of the risk of IBD-associated cancers, such as CAC, combined with advanced therapeutics, may lead to tremendous improvements in patient QOL in the near future.

In this review article, our recent achievements in discovering biomarkers to predict cancer risk are introduced. Ultimately, models based on combinations of genotype and gene expression data referenced with clinical, biochemical, and serological data may permit the development of tools for individualized risk stratification and efficient treatment selection, as well as complete rescue from complications, including CAC, in the near future[9].

Molecular pathogenesis of colorectal cancer and CAC

Chronic inflammatory diseases are associated with cancer incidence, which is dependent on the duration and severity of the diseases. For example, Barrett's esophagus is relevant to esophageal cancer, chronic *Helicobacter pylori*-associated chronic atrophic gastritis is relevant to gastric cancer, and UC or CD are relevant to CAC. These are well-acknowledged examples that support a connection between gut inflammation and cancer. In fact, it has been reported that patients with IBD are at enhanced risk of colorectal cancer (CRC): approximately 15% of CRC patients have related IBD etiology[10]. Although general carcinogenesis is a multi-factorial process that combines accumulation of genetic mutations, post-translational modification, and cell-matrix reciprocal action, inflammation-prone carcinogenesis is somewhat different[11]. The utility of biomarkers for CAC can be extended to permit earlier detection of dysplasia; therefore, the targeted manipulation of biomarkers might lead to advances in cancer therapies and cancer preventions, and may prove to be effective in reducing the development of CAC, with clinical interventions such as blocking agents or

endoscopic treatments. Regarding molecular aspects, the mechanism of CAC in IBD differs from CRC ,which is a well-known adenoma-to-carcinoma sequence. CAC appears to take place from either flat dysplastic tissue or dysplasia-associated lesions or masses[12]. The major pathways of sporadic CRC and CAC comprise chromosomal instability, hypermethylation and microsatellite instability; however, CAC shows inflamed colonic mucosa before histological changes of dysplasia or cancer. Patients with IBD have a high risk of CAC following diagnosis, and patients have common symptom, such as colitis[13]. The risk of CAC is increased in younger patients, those with more extensive colitis, those with concomitant primary sclerosing cholangitis, and a family history of CRC[14]. Most cancers show no high risk related to proctitis; however, increased in pancolitis, *i.e.*, left-sided colitis, carries an intermediate cancer risk[15]. Patients with CD and UD have the same risk of CRC and the prevalence in the US is greater than 200 cases per 100000, representing a total of between 1 and 1.5 million patients with IBD. Fortunately, the incidence of CAC is lower than CRC in the United States and other western countries[16]. A biological background for the high risk of CRC in IBD gives a one-sided interpretation, patients with high levels of inflammatory mediators production may progress to CAC. The key signal of IBD-induced carcinogenesis is inflammatory cytokines induced by mucosal and immune cells in the gut. The key molecules of inflammation, including nuclear factor kappaB (NF-κB) and cyclooxygenase-2 (COX-2), are important links between inflammation and cancer. Recently, other factors, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) -induced signaling, have been proven to induce cancer development in animal models of CAC[17]. Based on these aspects of molecular carcinogenesis, we have tried to apply biologicals such as infliximab to neutralize TNF-α, a proton pump inhibitor based on mechanisms including the inhibition of NF-κB as well as attenuation of oxidative stress, and aspirin/celecoxib to inhibit cancer-prone COX enzymes. As expected, all of these efforts to block inflammation-promoted carcinogenesis efficiently prevented CAC in our mouse model experiments[18,19]. The recent descriptions of epigenetic alterations, in particular alterations in DNA methylation, that have been observed during inflammation and inflammation-associated carcinogenesis, led us to explore nutritional interventions as a means of targeting and correcting epigenetic oncogenic abnormalities, as a form of CAC prevention[20].

Prediction of CAC

Patients with long-standing UC and CD are at increased risk of developing CRC, and patients with small intestinal CD have a high risk for developing small bowel adenocarcinoma. Unlike the sporadic CRC that can develop in those with IBD, CAC development is intimately associated with IBD. In those with IBD, CAC results from a process that is believed to begin with mutagenic benign inflammation that develops into indeterminate, low-grade and high-grade dysplasia, and eventually to carcinoma. Regarding the risk factors predisposing to carcinoma in IBD, the risk is increased depending on duration, severity of colitis, presence of sclerosing cholangitis, degree of inflammation and family history of CRC. Evidence-based medicine advises that patients with colitis should be kept under surveillance colonoscopy after diagnosis for 8 to 10 years. As surveillance guidelines for early detection of CAC, the general approach of periodic endoscopic examinations and systematic random biopsies of involved mucosa is generally recommended[21]. Recently, advanced colonoscopic techniques, including narrow band imaging, chromoendoscopy and confocal microendoscopy, have been used to identity abnormal areas in targeted, but not random, biopsies, and biomarkers could be adopted for high resolution endoscopy[22]. Although medications such as aminosalicylates, folic acid and ursodeoxycholic acid seem to be chemopreventive, potent preventive therapeutics, as well as surveillance of high risk patients through the use of potential biomarkers, would seem to be ideal[23].

Current Status Of Biological Markers For IBD

Serological markers for IBD are rapidly developing. However, the most studied antibodies, anti-*Saccharomyces cerevisiae* antibodies (ASCA) and atypical perinuclear antineutrophil cytoplasmic antibodies (P-ANCA), have limited sensitivity. Thus, the relationship between serological markers of disease pattern and phenotype may be of greater value than the use of serological markers as diagnostic tools. For example, patients with CD who have high titers of various serological makers have more serious small intestine disease than those with low titers of antibodies[24]. On the genetic level, application of a genome wide association study design in CD has provided new insights into the immunopathogenesis of CD, identifying links to genes of the innate and adaptive immune system[25]. One patient had CD associated with gene mutations of *NOD2* and the *ATG16L1* autophagy gene, both of which affect the intracellular processing of bacterial components. In addition, genetic variation of the IL-23 receptor, *STAT3* and *NKX2*-3 genes, were associated with CD and UC in Asian patients. Although comparative analyses of gene associations between CD and UD can identify unique mechanisms of immunopathogenesis of IBD, such results have limited applicability in real-world clinical settings because of ethnic, racial, and environmental differences in the samples studied. Since the advent of the concept of proteomics, a plethora of proteomic technologies have been developed to study proteomes. In IBD, several studies have used proteomics to better understand the disease and discover molecules that could serve as therapeutic targets. The advance of proteomic technologies will have an important effect on the development of new biomarkers for IBD[26]. Further advances in proteomic technologies have allowed us to use label-free quantification to detect biomarkers in various IBD patients for the first time. The results are

expected to provide additional insights in to the molecular biomarkers of IBD that may be used in predicting responses to treatment[27].

Classic serological and fecal markers in IBD

Currently, diagnosis of IBD from the blood and stool of patients represent reliable and quantitative tools to clinicians[28]. The C-reactive protein (CRP) and fecal-based leukocyte markers, calprotectin (Cal) or lactoferin (Lf), can help clinicians to assess disease activity and to distinguish IBD from non-inflammatory diarrhea and simple colitis. Serological tests including both ASCA and P-ANCA can be used to determine the current status and risk of IBD[29]. The progression of IBD and inflammatory processes are assessed by tests for CRP and the erythrocyte sedimentation rate (ESR). In addition, clinicians might measure the levels of drug metabolites and antibodies against therapeutic agents that aim to determine why patients do not respond to treatment and to select alternative therapy. The advantages of using the fecal markers including, Cal or Lf, are their ease of detection and use of an inexpensive ELISA technique, as well as their long-term stability in feces[30]. However, several limitations have been associated with these classical serological and fecal markers in IBD. The ESR technique is simple to perform, widely available, and inexpensive; however, it has several disadvantages, such as a concentration that depends on age, several confounders, and the use of certain drugs[31]. Several factors can affect the utility of CRP, including long half-life and prolonged latency period after changes in chronic IBD. Determination of fecal Cal or Lf markers is very helpful into diagnosis chronic IBD, while other GI diseases, ischemic colitis, and non-steroidal anti-inflammatory drug-associated intestinal damage show greater leukocyte elimination in feces[32]. In spite of 80%-100% diagnostic accuracy levels, fecal markers are not specific for IBD and may be elevated in a range of organic conditions. To compensate for these limitations, Langhorst *et al*[33] evaluated fecal levels of PMN-elastase (PMN-e) in addition to the aforementioned markers and concluded the IBD and IBS can be discriminated by the fecal markers Cal, Lf, and PMN-e. However, these fecal markers have shown a similar capacity to indicate endoscopic pathology, and are more efficient for diagnosis than CRP. Therefore, the combined diagnosis using fecal markers and CRP with disease-specific activity index will be very useful when assessing endoscopic inflammation in UC. It should be noted that these fecal markers were proven to be very efficient in diagnosing IBD as well as in predicting impending clinical relapse in pediatric patients with IBD[34]. Despite the benefits that can be derived from these serum and fecal biomarkers, there remains considerable room for improvement regarding disease prediction and prognosis assessment, as none of them can be applied to predict future risk of CAC development in IBD.

Proteomic biomarkers in IBD

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and surface-enhanced laser desorption/ionization (SELDI)-TOF MS, have become popular methods recently for the analysis of macromolecules of biological origin such as tissues, serum, or plasma[35]. MALDI-TOF MS is used in clinical medicine to identify disease markers in combination with classic protein gel analysis (1-D) and two dimensional gel electrophoresis separation, accomplished through either peptide mass fingerprinting or peptide sequence tag (2-D) analysis followed by a data base search using proteome blot analysis software[36]. Using these applications, evaluation of samples by MALDI-TOF MS can give novel data regarding peptides present at high molecular mass and may therefore be valuable to assess potential disease markers of IBD. For example, Nanni *et al*[37] determined serum proteins of 22 healthy subjects and 41 patients with IBD (15 CD, 26 UC) extracted with reversed-phase (C18) and subsequently performed MALDI-TOF MS . The results of serum protein profiles showed the highest overall prognosis capability (96.9%) of identifiable protein biomarkers involved in IBD discrimination . Similarly, in a study by Liu *et al*[38], serum proteins from 74 CRC samples compared with 48 healthy samples were applied to SELDI-TOF MS using a ProteinChip reader. The diagnostic pattern could distinguish samples according to status of CRC from normal samples with sensitivity and specificity of 95% by independent analysis of the samples. These two studies demonstrated the high potential for biomarker discovery in patients with IBD or CRC in clinical settings, and further clinical validation in large patient cohorts is expected to promote the use of novel biomarkers in clinical practice[39]. In addition to protein identification, 2-D difference gel electrophoresis (2D DIGE) is good method protein quantification; however, isobaric tags for relative and absolute quantification (iTRAQ) and stable isotope labeling by amino acids (SILAC) are the best methods developed recently. Sample preparation is important to the success or failure of such analysis and subcellular fractionation can be used to give more specific protein localization analysis than total cellular proteins[40-42]. In our recent study[43], we applied advanced technologies such as iTRAQ and SILAC for label-free quantification in samples obtained from a mouse model of UC and CAC to identify potential biomarkers for cancer-prone inflammation in IBD and to evaluate empirical therapeutics.

PROTEOMIC BIOMARKER DISCOVERY FOR CAC: A CLASSICAL PROTEOMIC APPROACH

Multiple chemical and biological systems, including intestinal tissue, its associated immune system, the gut microbiota, xenobiotics, and their metabolites, meet and interact to form a tightly regulated state of tissue homeostasis. Disturbance to this state of homeostasis can cause IBD as well as CAC through intercalated multi-factorial

mechanisms. Many strong pathological and mechanistic correlates exist between mouse models of CAC and the clinically relevant situation in humans, allowing for the use of systems biology approaches[44]. Furthermore, the close proximity of colonic tumors to the myriad of intestinal microbes, as well as the instrumental implication of microbiota in IBD, introduces microbes as new factors capable of triggering inflammation and possibly promoting CAC, necessitating high throughput metabolomic approaches[45]. Additionally, a detailed understanding of these interactions may also provide a means of preventing CAC[46]. In a small study, Watanabe *et al*[47] performed a low density array analysis of 149 genes implicated in CAC and identified 20 genes showing differential expression between UC- and non-UC-associated CAC, including cancer-related genes such as *CYP27B1*, runt-related transcription factor 3, sterile alpha motif domain-containing protein 1, EGF-like repeats and discoidin I-like domain 3, nucleolar protein 3, *CXCL9*, integrin beta2, and *LYN*. Colliver *et al*[48] demonstrated that 392 transcripts showed differential expression during progression from UC to CAC. Both dysplasia and CAC showed 224 transcripts in common and it was concluded that some genes showed same modification both in dysplasia and CAC, signifying that they might be related to tumor initiation and progression. Following these studies, a host of potential biomarkers have been reported in the literature, including cytokeratin 7/20[49], a-methylacyl-CoA-racemase[50], transgelin, a frame shift mutation in the TGF-β type Ⅱ receptor[51,52], HSP47[53], methylation of the estrogen receptor[54], association with certain HLA class Ⅱ alleles[55], DNA methyltransferase-1[56], 8-nitroguanine or 8-oxo-deoxyguanine[57-59], CCL20[60] and activation-induced cytidine deaminase[61]. We used our experimental animal model for colitic cancer, which was provoked with repeated bouts of UC, and an additional proteomic method based on 2-D electrophoresis and MALDI-TOF MS to analyze proteins related in CAC. In detail, 38 proteins were differentially expressed between CAC and healthy samples, using comparative 2-D electrophoresis analysis. Through validation studies, 27 proteins, including enolase, GRP94, HSC70 prohibitin and transgelin, were identified. Among these identified proteins, the downregulation of transgelin in mouse colitic cancer was supported by western blotting and immunohistochemistry. Moreover, transgelin was significantly decreased in colon tumors compared with non-tumorous regions in humans, implying that reduced levels of transgelin could be a good biomarker for CAC[62].

BIOMARKER DISCOVERY FOR CAC: THE NEXT GENERATION OF PROTEOMICS

Currently, knowledge of proteomics is important and provides researches with complicated label and label-free techniques. This next generation of proteomics may provide effective perspectives in the diagnosis and treatment of gastrointestinal disease and allow for translation of proteomics from the bench to the bedside. Currently, proteomic studies focus not only in the identification of proteins in a sample, but also the quantification of them. Various protein expression profiles are examined because they provide useful information, especially in clinical proteomics, involving molecular targets related to specific diseases. The technology for proteomics study is continuously developing, and both of labeled and label-free methods have their several advantages. Currently, label-free and isotope-label techniques are used to study proteins quantitatively. The label-free approach, combining spectral and signal quantitation, for identifying amino acids provides accurate relative protein expression data and is an easy way to determine quantitative information. However, this strategy is subject errors from variation in protein preparation that may be reduced when various stable isotopes are inserted in the specimens to make protein isotopomers, which have different spectra according to their different masses. Therefore, several metabolic labeling strategies that apply stable isotopes to minimize error have been developed recently and have been applied in animal models.

Biomarkers to predict CAC risk discovered via label-free quantification analyses

A comparative label-free quantification analysis was conducted in eight patients with UC, eight patients with CD and eight patients with irritable bowel syndrome (IBS). Colonic tissue biopsies were obtained during colonoscopy after written consent and stored in a deep freeze until the assay. Using an Agilent HPLC-Chip 6520 Q-TOF MS system and a label-free quantitative technique (IDEAL-Q v1.0.6.3), signal pathway analysis associated with carcinogenesis was conducted to discover potential biomarkers in CAC (Figure 1). The analysis was conducted according to the degree of intestinal inflammation, type of IBD, and extent of inflammation. To compare against the analysis from IBS samples, the proteins implying CAC risk were isolated (Figure 2A and B). As seen in Figure 2A, 22 significant proteins were found to be potential biomarkers predicting CAC risk in patients with UC . Figure 2B shows the 19 proteins found to be potential biomarkers for CAC risk in patients with CD. Further analysis yielded four important protein biomarkers: proteoglycan 2 (PRG2), S100A6 (calcyclin), ribosomal protein L18 (RPL18), UDP-glucose dehydrogenase (UGDH) as potential target proteins and predictive biomarkers for CAC risk in IBD (Figure 3). PRG is a major component of the animal extracellular matrix and has been shown to be involved in the differentiation process across the epithelial-mesenchymal axis. It is a potential biomarker inferred principally through its ability to bind growth factors and modulate their downstream signaling; malignant tumors have their individual characteristic PRG profiles closely associated with their differentiation and biological behavior. PRG2 has further been implicated as a biomarker for neuropathic pain attributable to advanced pancreatic cancer[63] in an animal model of CAC[27], and as an inflammation related gene of several cancers, including prostate,

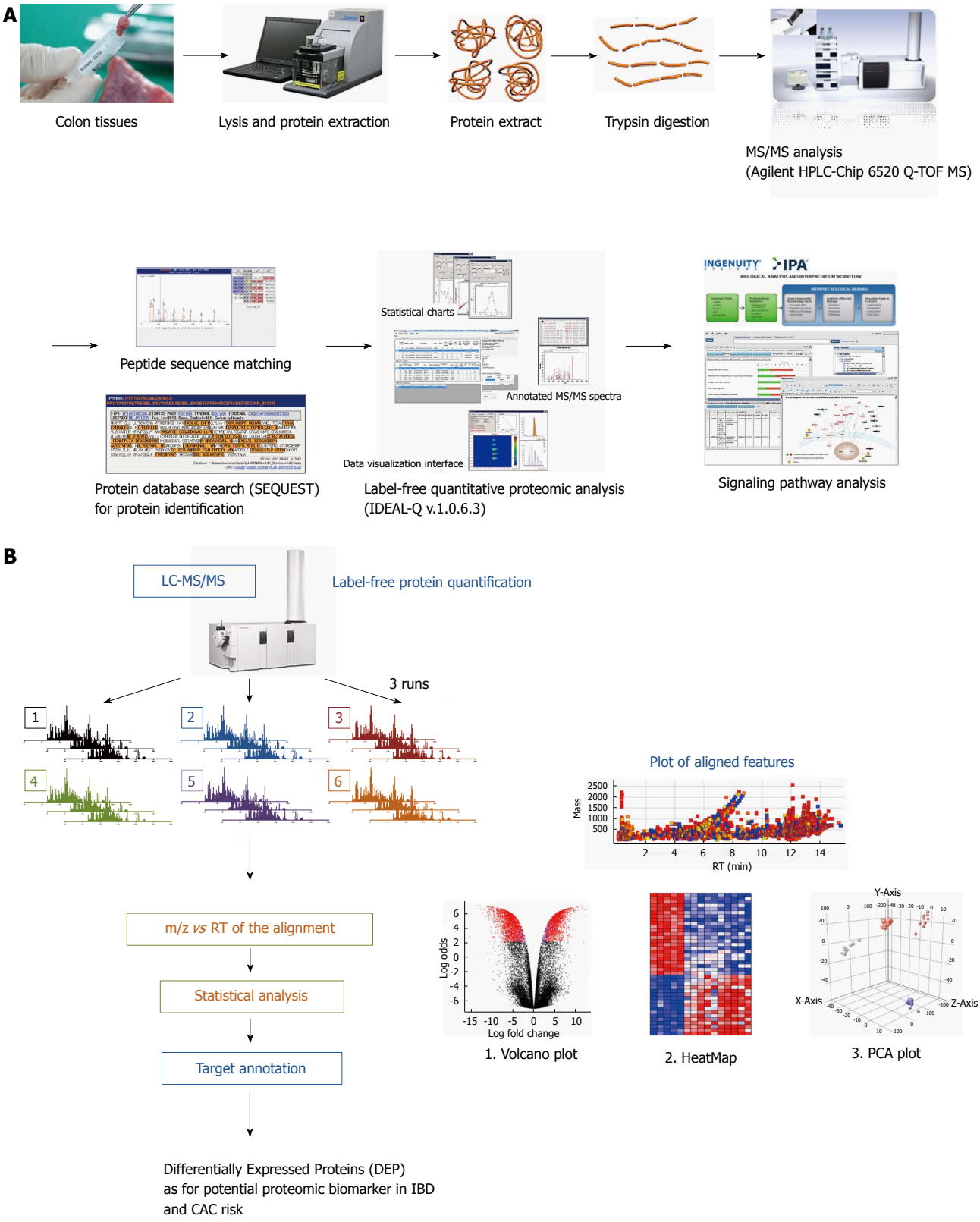


Figure 1 Schematic presentation showing proteome analysis to discover potential biomarkers and label-free quantification analysis in inflammatory bowel disease. A: Applying the label-free quantification method to discover proteomic biomarkers in patients with different types and different stage of inflammatory bowel disease (IBD). Comparative analysis was done in eight patients with ulcerative colitis (UC), eight patients with Crohn's disease (CD) and eight patients with irritable bowel syndrome (IBS). Biopsied colon tissues were obtained during colonoscopy after written consent, and stored in a deep freeze until assayed. Using Agilent HPLC-Chip 6520 Q-time-of-flight mass spectrometry (TOF-MS) and label-free quantitative proteome analysis (IDEAL-Q v1.0.6.3), significant signal pathway analysis was done. In the current review, the analysis done according to the degree of intestinal inflammation, type of IBD, and extent of inflammation from 24 patients, eight from non-IBD normal patients; *i.e.*, IBS patients, eight from patients with UC, and from patients with CD; B: Label-free protein quantification scheme for potential biomarker for colitis-associated cancer (CAC) risk in 16 patients with IBD.

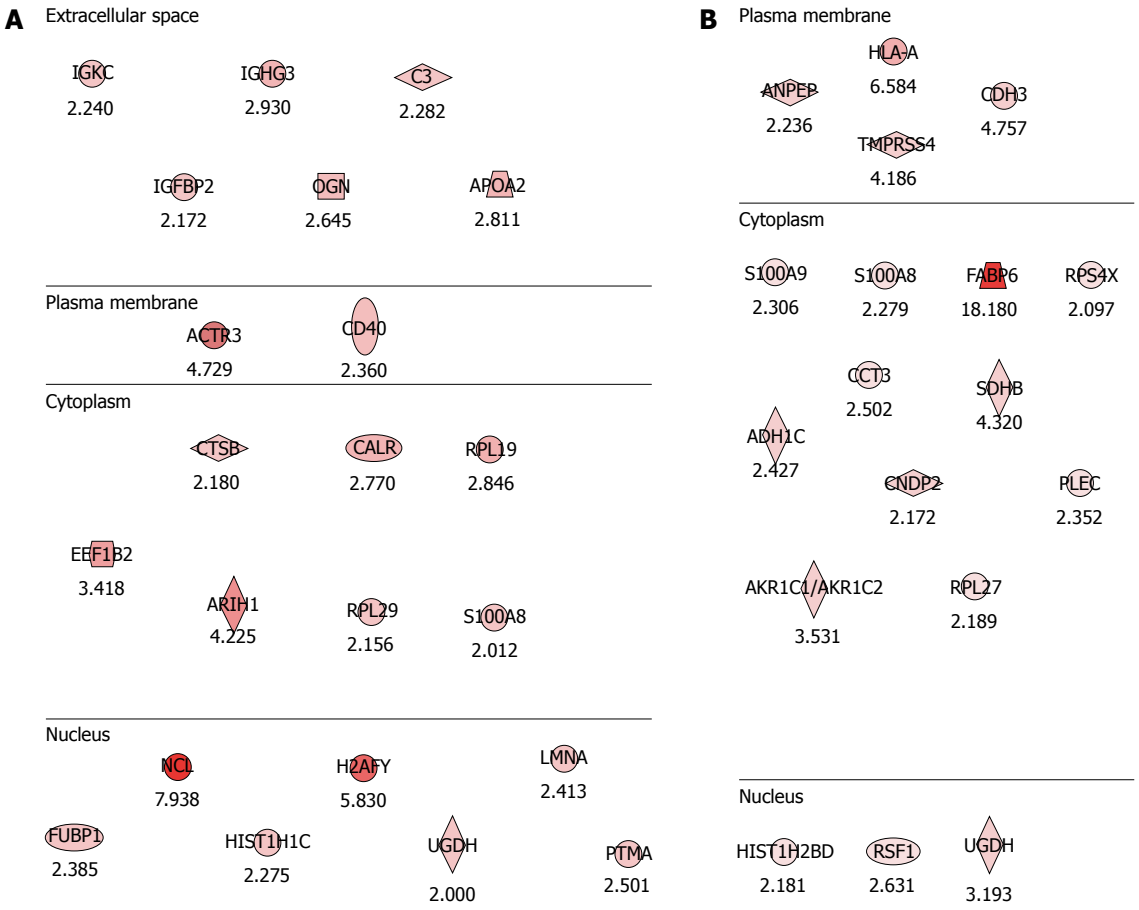
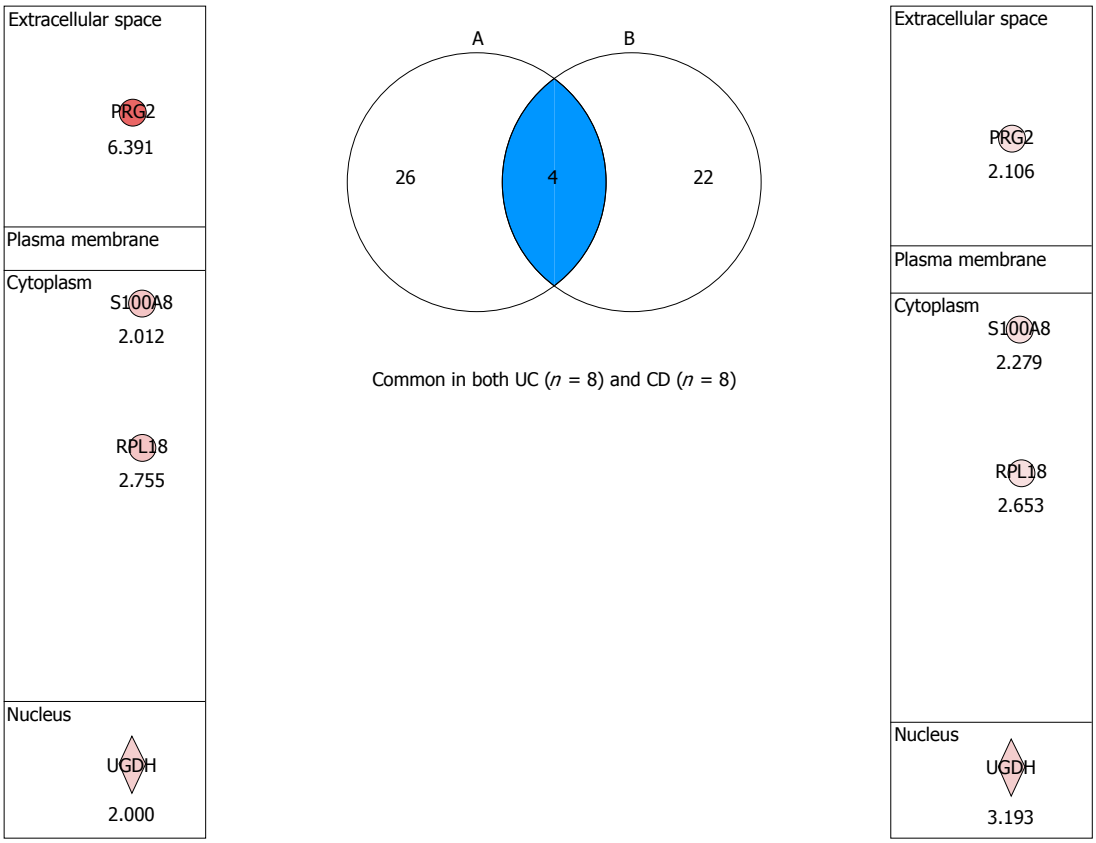


Figure 2 Potential proteomic markers signifying colitis-associated cancer risks in inflammatory bowel disease. A: Proteomic markers for colitis-associated cancer (CAC) risk in patients with ulcerative colitis (UC). The analysis was performed according to the degree of intestinal inflammation, type of inflammatory bowel disease and extent of inflammation. Compared with the analysis of CAC, 22 significant potential biomarkers of CAC risk were obtained from patients as p with UC, six biomarkers existing in the extracellular space, two biomarkers at the plasma membrane, seven are from the cytoplasm, and seven are nuclear proteins; B: Proteomic markers for CAC risk in patients with Crohn's disease (CD). Eighteen potential biomarkers for the risk of CAC were identified in patients with CD. Four were from the plasma membrane, 11 from the cytoplasm, and three from the nucleus.

lung, CRC[64] and pancreatic cancer[65]. The expression of S100 calcium binding protein A6 (S100A6) is upregulated in proliferating and differentiating cells[66], and has been reported to be a possible biomarker for hepatocellular carcinoma[67,68], pancreatic cancer[69], acute lymphoblastic leukemia[70], CRC[71] and breast cancer[72]. RPL18 is a 60S ribosomal protein expressed in stem cells[73] and during adipogenesis[74] UGDH has been suggested as biomarker for cancer metabolism. The oxidation of UDP-glucose is catalyzed by UGDH to generate UDP-glucuronic acid (UDP-GlcA), a precursor of glycosaminoglycans (GAGs). Wang *et al*[75] showed decreases in the expressions of UGDH, UDP-GlcA and GAG expression after treatment with a UGDH-siRNA in HCT-8 colon cancer cells and concluded that UGDH could be a new target for CRC clinical treatment[76]. Additionally, UGDH has been identified as a potential biomarker for prostate cancer[77,78], hepatocellular carcinoma[79] and breast cancer[80].

Biomarkers to predict CAC risk discovered via label-based protein quantification analyses

Proteomic techniques with blood and biopsy provide reliable and accurate tools that provide support to clinicians in the diagnosis and treatment of IBD. For example, clinically meaningful biomarkers may be used in the differential diagnosis of CD and UC, or as predictors of treatment responses. Tandem mass spectrometry (MS/MS) is commonly used proteomic analysis. However, various workflows are possible for peptide analysis before MS/MS, as well as bioinformatics, to identify peptides, for which 2-D electrophoresis and subsequent MS, liquid chromatography-MS, 2D DIGE, and iTRAQ are under development. In our previous publication[43], the present status and perspectives regarding these developed proteomic methods were discussed, with descriptions of examples of new biomarkers for the diagnosis, treatment and prognosis of IBD and CAC in mouse models and in humans. In detail, we showed new concepts and technologies of proteomics, such as protein identification and proteome coverage, as determined by iTRAQ with different shotgun proteomic methods in samples from an animal model of CAC that used repeated oral administration of dextran sulfate sodium (DSS) to induce CAC. As previously reported, iTRAQ protein quantification analysis identified fibrinogen beta, prohibitin, transgelin, Hsc-70-interacting protein, suppression of tumorigenicity 13



Symbol	Entrez gene name	Fold change (CD)	Fold change (UC)	Location	Type (s)
PRG2	proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein)	2.106	6.391	Extracellular space	Other
RPL18	ribosomal protein L18	2.653	2.755	Cytoplasm	Other
S100A8	S100 calcium binding protein A8	2.279	2.012	Cytoplasm	Other
UGDH	UDP-glucose 6-dehydrogenase	3.193	2.000	Nucleus	enzyme

Figure 3 Proteomic markers for colitis-associated cancer risk in patients with both ulcerative colitis and Crohn's disease. After analyzing signaling pathways from label-free quantitative analysis, four important proteome biomarkers were identified: proteoglycan 2 (PRG2), S100 calcium binding protein A8 (S100A8), ribosomal protein L18 (RPL18), and UDP-glucose dehydrogenase (UGDH), all of which showed fold changes. Validation is ongoing to investigate these biomarkers for predicting colitis-associated cancer risk in patients with inflammatory bowel disease. UC: Ulcerative colitis; CD: Crohn's disease.

(ST13), a TKL kinase of the MLK family, dual leucine zipper kinase (MKL1), actin, beta, protein-coding gene (*ACTB*), ubiquitin carboxyl-term, esterase L3 (UCHL3), coronin, actin binding protein, 1A (CORO1A), hypoxanthin-guanine phosphoribosyltransferase (HPRT), glutathione peroxidase 1 (GPX1), estrogen receptor 1 (ESR1), transcriptional repressor protein 1 (YY1), transcription activator1, ATP-dependent helicase SMARCA4 (BRG1), brahma gene (BRM) and ornithine aminotransferase (OAT) as potential biomarkers for CAC in the DSS-induced colitic cancer model[43].

CONCLUSION

MALDI imaging mass spectrometry (IMS) is a new technology to analyze small peptides in various samples and is a novel tool for studying molecular mechanisms in biological tissues. Recent studies have demonstrated considerable diagnostic and prognostic value, which should be applicable to clinical settings in the near future[81,82]. However, one challenge associated with the use of MALDI-IMS in the identification of potential biomarkers involves the systematic identification of peptides introduced in the MALDI matrix with association of top-down and bottom-up analysis[83]. IMS will be investigated without target-specific reagents to identify new markers for the diagnosis, treatment, and prognosis of CAC, as well as the determination of effective therapies. In the near future, an era of tailored medicine will provide for diagnostic algorithms that include molecular parameters for the detection of early disease and treatment algorithms guided by predicting the individual course of the disease. However, more trials focused on discovering proteomic biomarkers will be necessary to guide the treatment of IBD with more advanced levels of biologicals or molecular targeted therapeutics for inflammation. Using label-free quantification methods on biopsied tissue from patients with IBD, four potential biomarkers, PRG2, S100A6 (calcy-

clin), RPL18, and UGDH, have been discovered. Further validation of these potential biomarkers will be necessary to ascertain their clinical value.

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REVIEW

Complex interactions between microRNAs and hepatitis B/C viruses

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Abstract

MicroRNAs (miRNAs) are a class of small noncoding RNAs that post-transcriptionally regulate the expression of many target genes *via* mRNA degradation or translation inhibition. Many studies have shown that miRNAs are involved in the modulation of gene expression and replication of hepatitis B virus (HBV) and hepatitis C virus (HCV) and play a pivotal role in host-virus interactions. Increasing evidence also demonstrates that viral infection leads to alteration of the miRNA expression profile in hepatic tissues or circulation. The deregulated miRNAs participate in hepatocellular carcinoma (HCC) initiation and progression by functioning as oncogenes or tumor suppressor genes by targeting various genes involved in cancer-related signaling pathways. The distinct expression pattern of miRNAs may be a useful marker for the diagnosis and prognosis of virus-related diseases considering the limitation of currently used biomarkers. Moreover, the role of deregulated miRNA in host-virus interactions and HCC development suggested that miRNAs may serve as therapeutic targets or as tools. In this review, we summarize the recent findings about the deregulation and the role of miRNAs during HBV/HCV infection and HCC development, and we discuss the possible mechanism of action of miRNAs in the pathogenesis of virus-related diseases. Furthermore, we discuss the potential of using miRNAs as markers for diagnosis and prognosis as well as therapeutic targets and drugs.

Key words: MicroRNA; Hepatitis B/C virus; Host-virus interaction; Biomarker; Therapy

Core tip: Chronic hepatitis B virus or hepatitis C virus infection changed miRNA expression profiles at the tissue or serum level, and the altered miRNAs play pivotal roles in gene expression and replication of the viruses and the development of virus-related diseases. These findings suggest that miRNAs have the potential as novel diagnostic and prognostic biomarkers and therapeutic targets in virus-related diseases.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer-related death worldwide. Chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections resulting in chronic liver diseases are the major causative factors for HCC[1]. Numerous studies have showed that HCC develops through aberrantly activation of various signaling

pathways involved in cellular proliferation, differentiation, angiogenesis, and metastasis. But the precise molecular mechanisms underlying virus induced-HCC development and progression are still unclear.

MiRNAs are endogenous, small noncoding RNAs containing an average of 22 nucleotides that mainly regulate gene expression at the post-transcription levels through sequence-specific binding to the 3'-untranslated region (UTR), coding region or 5'-UTR of the target mRNA[2]. As most miRNAs guide the recognition of imperfect matches of target mRNAs, each miRNA can have tens to hundreds of different mRNAs targets and further one mRNA can be simultaneously regulated by multiple miRNAs. More than 60% of all human protein-coding genes were predicted to be under miRNA regulations, which enable miRNAs to have numerous regulatory roles in many physiological processes, such as cell proliferation, differentiation and apoptosis[3].

Due to their fundamental role in biological process, aberrant expression of miRNAs in the liver may be involved in pathological processes of various liver diseases. In fact, the specific deregulation of certain miRNAs, along with the roles of the miRNAs, was observed in liver diseases such as HBV/HCV infection, fibrosis, cirrhosis and HCC[4]. In this review, we will focus on the regulatory role of deregulated miRNA in HBV/HCV infection and HCC development. In addition, we briefly discuss miRNAs as potential diagnostic markers, prognostic markers and therapeutic targets for virus-related liver diseases.

REGULATION OF THE HBV/HCV LIFE CYCLE BY HOST MIRNAS

MiRNAs are reported to be involved in regulation of virus expression and replication mainly by binding directly to virus transcripts or targeting the related host genes[5,6]. Here, we will focus on a subset of miRNAs that are reported to affect gene expression and replication of HBV/HCV.

Role of miRNAs in HBV gene expression and replication

To systemically screen for host miRNAs that affected HBV replication, our lab employed a loss-of-function approach (transfecting chemically synthesized antisense oligonucleotides of 328 identified human miRNAs into HepG2.2.15 cells) and found that miR-199a-3p and miR-210 inhibitors could increase HBsAg expression and HBV replication without a significant effect on host cell proliferation. Further bioinformatics analysis and a GFP reporter assay validated that miR-199a-3p and miR-210 reduced HBV replication by binding to the HBV S protein coding region and pre-S1 region, respectively[7]. This is the first report that human miRNAs can directly target HBV genes. In addition, Potenza *et al*[8] reported that miR-125a-5p, a miRNA highly expressed in human liver tissue, was able to interfere with the translation of the HBV *S* gene. Wu *et al*[9] used four well-established target-prediction programs to predict the targets of human miRNAs in the HBV genome, and they found that let-7, miR-196b, miR-433, and miR-511 targeted the polymerase or S gene, miR-205 targeted the *X* gene, and miR-345 targeted the preC gene. Moreover, the target regions are conserved among different HBV clades, which implied that these miRNAs can be used in antiviral therapy. However, the anti-HBV activities of the miRNAs need further experimental validation. miR-15a/miR-16-1,MiR-20a and miR-92a-1 (two members of miR-17-92 cluster), and miR-224, were shown to suppress HBV replication *in vitro* possibly by directly binding to HBV genes[10-12].

MiRNAs can also modulate HBV replication by targeting HBV-associated host proteins. The study by Zhang *et al*[13] showed that miR-1 enhanced HBV replication by increasing HBV core promoter activity through augmenting farnesoid X receptor α expression. MiRs-372/373 stimulated the production of HBV proteins and HBV core-associated DNA in HepG2 cells by targeting nuclear factor I/B[14]. MiR-501 was recently reported to promote HBV replication in part by targeting HBXIP, an inhibitor of HBV replication in HepG2.215 cells[15]. MiR-141 suppressed HBV replication by down-regulating peroxisome proliferator-activated receptor alpha, a positive transcription factor of HBV[16]. MiR-122, an abundant liver-specific miRNA, could inhibit gene expression and replication of HBV *in vitro via* binding to highly conserved regions of the mRNA for the viral polymerase and the 3'-untranslated region of the mRNA for the core protein[17]. Further mechanism studies showed that miR-122 suppressed HBV replication partially by modulation of p53-mediated inhibition of HBV replication through down-regulation of cyclin G1[18,19]. MiR-155 mildly inhibited HBV infection in human hepatoma cells by suppressing suppressor of cytokine signaling 1 (SOCS1) expression and subsequently promoting JAK/STAT(signal transducer and activator of transcription) signaling pathway, which leads to enhanced innate antiviral immunity[20] (Figure 1A).

Role of miRNAs in HCV expression and replication

In contrast to the inhibitory role on HBV replication, miR-122 is essential for HCV RNA replication. Jopling *et al*[21] found that sequestration of miR-122 led to a marked loss of HCV RNAs *in vitro*. Further study showed that the interaction between miR-122 and two binding sites in the 5'-noncoding region of the HCV genome were essential for HCV RNA maintenance. The binding of miR-122 to the HCV genome could also stimulate HCV translation by enhancing ribosome binding to the viral RNA[22]. However, results from the double-binding-site mutation and the IRES mutation assays showed that miR-122 may affect viral replication at other steps of the viral life cycle[23]. Expression of miR-122 in non-hepatic cells could facilitate efficient viral replication, which further supports the finding that miR-122 is essential for HCV replication[24,25]. Further structure and function

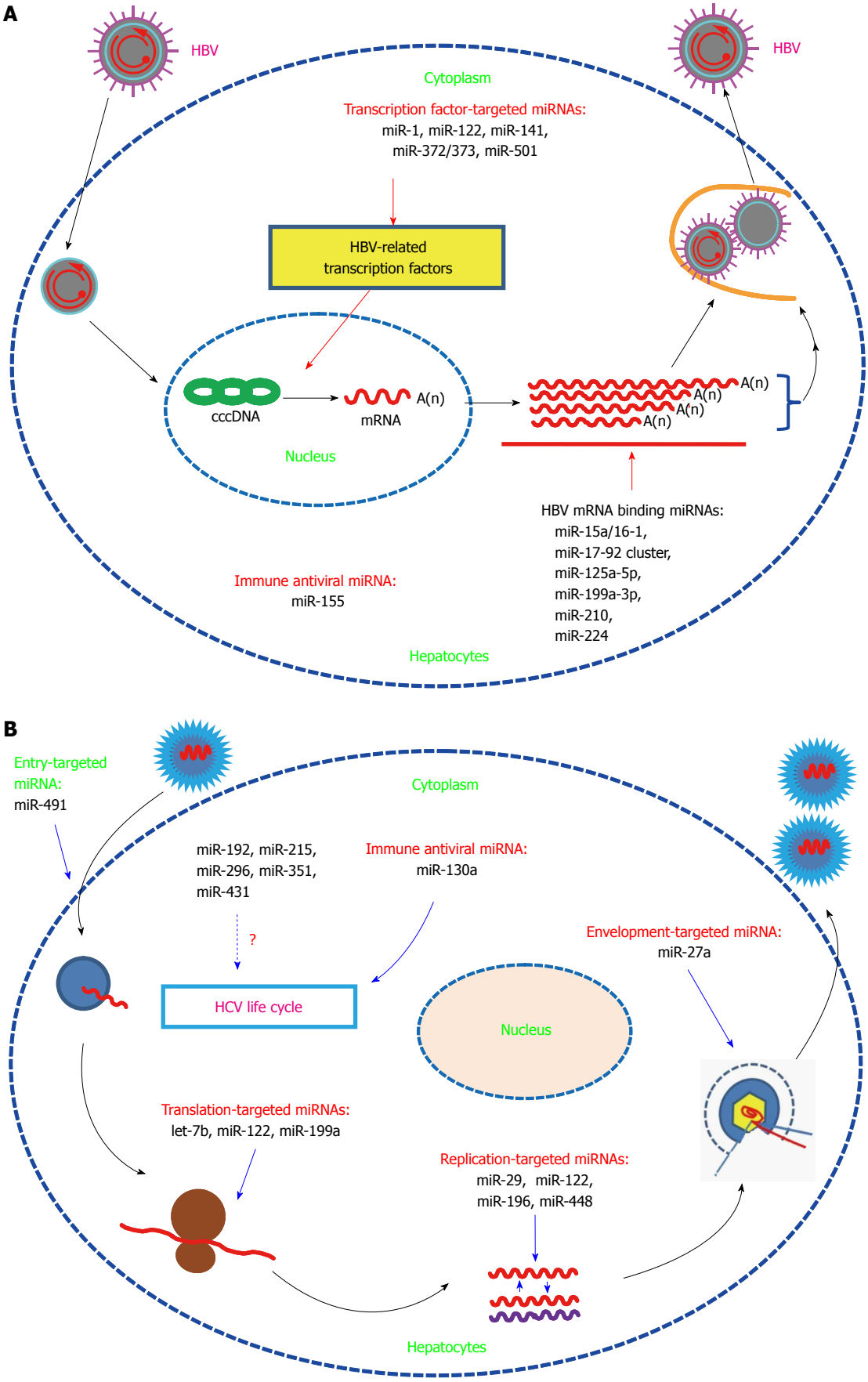

Figure 1 Summary of cellular miRNAs effect on hepatitis B virus and hepatitis C virus replication. A: The miRNAs which regulate hepatitis B virus (HBV) replication through targeting transcription factors known for HBV transcription, regulating immune, and direct targeting viral transcripts were indicated; B: The miRNAs which regulate hepatitis C virus (HCV) replication through targeting entry, translation, replication, envelopment, and regulating immune were indicated.

analysis showed that nearly all nucleotides in miR-122 were involved in binding to the second binding site, and this additional interaction enhanced HCV replication[26]. MiR-122 could bind to HCV RNA in association with Ago2 to protect the viral genome from 5'-exonuclease activity and thus stabilize the HCV genome and slow its decay[27]. A recent study showed that the formation of a stable ternary complex between two copies of miR-122 and the HCV 5'-UTR could stabilize the HCV genome, which also implied that miR-122 had additional functions in the viral life cycle[28]. In addition to direct binding to the HCV genome, miR-122 may promote HCV replication through other pathways. Shan *et al*[29] found that suppression of miR-122 with an miR-122-specific antagomir decreased HCV replication and increased HO-1 expression, thus suggesting that miR-122 may promote HCV replication in part by decreasing heme oxygenase 1(HO-1) expression. HO-1, a key enzyme with anti-oxidant and anti-inflammatory activities, can suppress HCV RNA replication[30].

Although the specific mechanism of miR-122-supported HCV replication needs further exploration, the above studies indicated that miR-122-based therapy may be useful in limiting HCV infection. Silencing of miR-122 with a locked nucleic acid (LNA)-modified oligonucleotide (SPC3649) complementary to miR-122 resulted in universal antiviral activity against diverse HCV genotypes *in vitro* and led to long-lasting suppression of HCV viremia in chronically infected chimpanzees with no evidence of viral resistance or side effects[31,32]. In 2009, the miR-122 inhibitor miravirsen was applied to phase 1 clinical trials by Santaris Pharma to test its safety. The data demonstrated that miravirsen was well tolerated and has no dose-limiting toxicities[33]. In 2010, Santaris Pharma initiated phase 2a clinical trials and showed that miravirsen was efficient in reducing HCV RNA in patients with chronic HCV genotype 1 infection. Moreover, this antiviral effect was long-lasting, and no detectible adverse events or escape mutations were observed[34]. However, it has been reported that miR-122 could inhibit nuclear factor (NF)-κB activation and subsequently its downstream pro-inflammatory events[35]. So silencing of miR-122 may augment liver inflammation. One study showed that interferon (IFN)-β treatment leads to a significant reduction in the expression of miR-122[36]. Other studies showed that during chronic HCV infection, patients with decreased pretreatment liver miRNA-122 levels responded poorly to interferon therapy[37-39]. All these issues should be considered when using miravirsen alone or in combination with IFN therapy. Furthermore, the suppression effect of miR-122 on HBV should be taken into consideration when treating HCV infection with miravirsen in HBV and HCV co-infected patients.

In contrast to the inhibitory effect on HBV, HCV infection-induced miR-141 could promote efficient HCV replication in primary human hepatocytes, which was dependent on miR-141 mediated suppression of the tumor suppressor gene DLC-1[40]. MiR-192, miR-215, and miR-491, which were found to be altered after HCV infection, could enhance HCV replication. Among these miRNAs, miR-491 promoted HCV replication through the PI3 kinase/Akt pathway which may enhance entry of HCV into cells, while the mechanisms for the other two miRNAs were not mentioned[41]. In contrast to the enhancing capabilities of the above-mentioned miRNAs, miR-199a inhibited HCV replication in a cell culture system by directly targeting to the sequence in domain II of the IRES region in the HCV 5'-UTR[42]. MiR-196 inhibited HCV replication *in vitro* mainly through repressing the expression of Bach1, a basic leucine zipper mammalian transcriptional repressor of HO-1[43]. MiR-29 was down-regulated in both hepatocytes and hepatic stellate cells after HCV infection, and function analysis showed that miR-29 overexpression reduced HCV RNA abundance and inhibited collagen and extracellular matrix expression[44]. Therefore, miR-29 has potential as a therapy tool by inhibiting HCV replication and decreasing fibrosis. Expression of let-7b markedly suppressed HCV replication and down-regulated HCV accumulation and had a synergistic inhibitory effect on HCV infection with IFN-α-2a[45]. Although bioinformatics analysis identified conserved binding sites for let-7b among various HCV genotypes within the coding region of NS5B and the 5'-UTR of the HCV genome, mechanism studies showed that let-7b-mediated suppression of HCV RNA accumulation was independent of translation inhibition. Let-7b represents the first miRNA with a target site in the coding region of the HCV genome. Interestingly, a recent study showed that let-7b was regulated by IFNα and IL-28B and inhibited HCV replication and viral protein translation by targeting host factor insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1)[46]. HCV-induced miR-130a inhibits HCV replication/production by restoring host innate immune responses and/or down-regulating pro-HCV miR-122[47,48]. MiR-27a induced by HCV infection and lipid overload could in turn repress HCV infection and lipid storage *in vitro*. This negative feedback loop between miR-27a and HCV may be beneficial for immune escape and establishment of persistent HCV infection[49]. Pedersen *et al*[36] found that IFNβ-induced miRNAs miR-196, miR-296, miR-351, miR-431 and miR-448 could substantially attenuate HCV replication. Among them, miR-196 and miR-448 may inhibit HCV replication by directly targeting the HCV genomic RNA (Figure 1B).

Role of virus-encoded miRNA on the virus itself

To date, no HCV-encoded miRNAs have been reported, which may be due to the nature of RNA viruses or to the limitations of current detection technology. By using computational approaches, Jin *et al*[50] found that HBV putatively encodes only one candidate pre-miRNA. The presence of the mature form of the predicted miRNA in HBV-infected patients was validated by Northern blot. Further target search results showed that there was only one potential viral mRNA target, which suggested that

HBV has evolved to produce a miRNA to regulate its own replication. Studies investigating the specific function of this viral miRNA are in progress.

HOST MIRNA EXPRESSION AFTER HBV OR HCV INFECTION: DEREGULATION, FUNCTIONS AND MECHANISM

Increasing evidence showed that HBV or HCV has evolved various strategies to evade host immune surveillance and establish a persistent chronic infection, which includes modulating the expression of host miRNAs. The alteration of the miRNA expression profile by HBV or HCV infection, the possible mechanism responsible for miRNA deregulation, and miRNA roles in virus-related liver pathogenesis are reviewed.

MiRNA expression profiles after HBV infection and miRNA functions

Many reports have examined miRNA expression alteration in cell culture, HCC liver tissue and serum samples after HBV infection by miRNA-based microarray or sequencing. The cell line HepG2.2.15, which stably expresses HBV, and the parental cell line HepG2 are often used to compare the effect of HBV on miRNA expression. MiRNA microarray analysis showed that 11 miRNAs were up-regulated and 7 miRNAs were down-regulated in HepG2.2.15 cells compared to HepG2 cells[51]. After this initial report, additional studies have compared the miRNA expression profile in these two cell lines by miRNA array[14,52,53]. Among the up-regulated miRNAs, miR-199a, miR-210, miR-371/372, miR-501, and the miR-17-92 cluster were involved in HBV replication[7,11,14,15]. The mechanism by which HBV regulates miRNAs is largely unknown. It has been reported that the viral protein HBx could transactivate a variety of viral and host genes, including miRNAs. To date, it has been reported that HBx modulated miRNA expression mainly through 3 pathways. First, HBx regulates miRNA expression at the transcription level *via* direct binding to the transcription factor essential for miRNA transcription or by modulating the transcription factor expression[54-56]. Second, HBx mRNA functions as a sponge that can specifically down-regulate miRNA *via* the miRNA target sequence in the viral RNA[10,57]. Third, HBx modulates pri-miRNA biogenesis by reducing the protein levels of the RNase III enzyme Drosha[58]. However, there is evidence in HBV-associated HCC that the key regulators of miRNA biosynthesis, including Drosha, DGCR8, Ago1, and Ago2, are frequently overexpressed[59]. Except for HBx, the other HBV proteins have not been reported to be directly involved in regulating miRNA expression. The specific mechanism will be discussed in the related reference. MiRNA array analysis of HBx-expressing versus control HepG2 cells showed that 7 miRNAs were significantly up-regulated and 11 were markedly down-regulated in HBx-expressing cells[60]. HBx-mediated down-regulation of let-7a promoted cell proliferation and hepatocarcinogenesis in part *via* up-regulation of STAT3. HBx could also reduce the levels of another let-7 family member, let-7i, which regulated CD59 expression and thus protected the HCC cells from complement-dependent cytotoxicity[61]. Tumor suppressor-like miR-15a/16 was first reported to be repressed by HBx-induced c-myc[62]. Two recent findings showed that HBx transcripts could directly lead to miR-15a/16 down-regulation *via* the so-called miRNA sponge effect[10,57]. Therefore, the virus gene could regulate one miRNA expression in different ways. Moreover, miR-15b, another member of miR-16 family, was also repressed by HBx and suppressed cell proliferation *via* directly targeting fucosyltransferase 2 (FUT2), which leads to decreased levels of Globo H[63]. A recent study showed that HBx down-regulated tumor suppressor miRNA miR-205 through inducing hypermethylation of miR-205 promoter, which may be another pathway by which HBx regulating miRNA expression[64]. Quantitative real-time PCR analysis showed that miR-101, one of the most abundantly expressed miRNAs in human normal liver, was reduced by HBx *in vitro*. Function analysis showed that down-regulated miR-101 promoted aberrant DNA hypermethylation, which contributes to hepatocarcinogenesis by allowing DNMT3A expression[65]. Another association study of genetic variations in miR-101-1 and miR-101-2 with HBV-related liver diseases showed that the single nucleotide polymorphism (SNP) in miR-101-1 was associated with the development of HCC and liver cirrhosis[66]. To date, it has been reported that SNPs in the 3'-UTR of three miRNA processing genes, miRNA encoding genes or binding sites are associated with miRNA deregulation in HCC[67-78]. MiR-29a was dramatically increased in p21-HBx transgenic mice, HBx-transfected HepG2-X (or H7402-X) cells and HepG2.2.15 cells. MiR-29a enhanced hepatoma cell migration by targeting phosphatase and tensin homolog (PTEN)[79]. HBx-induced miR-21 enhanced cell proliferation by targeting tumor-suppressor genes Programmed Cell Death Protein-4 (PDCD4) and PTEN[80]. One report showed that miR-148 was repressed by HBx to promote cancer growth and metastasis in a mouse model by activating hematopoietic pre-B cell leukemia transcription factor- interacting protein (HPIP)-mediated mTOR signaling[54]. The mechanism studies showed that HBx suppressed miR-148a expression by directly interacting with p53. However, another study by Yuan *et al*[81] reported that miR-148 is induced by HBx, and the up-regulated miR-148 stimulated hepatocellular growth and tumorigenesis by repressing PTEN and subsequent induction of β-catenin expression. The mechanism responsible for the HBx-mediated miR-148 induction is not mentioned in this study. The opposite effect of HBx on miR-148 in the latter report may be because the authors examined the miR-148 levels in stable HBx-expressing HepG2 cell lines and because the biological characterization of HepG2 cells have changed for HBx integration. The specific reason for the observation requires further

exploration. Carboxyl-terminal truncated HBx (Ct-HBx) is frequently over-expressed in HBV-associated HCC and plays a critical role in hepatocarcinogenesis[55]. Ct-HBx down-regulated a set of growth-inhibitory miRNAs that were often induced by its full-length counterpart. Moreover, Ct-HBx could directly bind to some of these miRNA promoters to repress transcription. All of the above-mentioned miRNAs regulated by HBx play important roles in tumor formation. Therefore, HBx, which has been implicated in HBV-related HCC pathogenesis, may perform its pro-carcinogenic function partly through regulating host miRNA expression. Recently, one report showed that HBV up-regulated miR-181a expression by enhancing its promoter activity, HBV induced-miR-181a promoted cell growth *in vitro* and tumor formation *in vivo* possibly by targeting transcription factor E2F5[82].

Jiang *et al*[83] showed that a large number of mature and precursor miRNA expression was increased in hepatitis B or C virus-positive, cirrhotic tissues compared to normal liver. This is the first report that hepatitis infection altered miRNA expression in liver tissues. Later, Ladeiro *et al*[84] examined the expression of 250 miRNAs in 46 benign and malignant hepatocellular tumors with 4 normal livers as controls with real-time PCR and found that miR-96 was specifically overexpressed in HBV tumors. Connolly *et al*[85] found that two known oncomiRs, the miR-17-92 polycistron and miR-21, were significantly increased in human HBV-positive HCCs and woodchuck hepatics virus-positive HCCs. Ura *et al*[86] analyzed the expression of 188 miRNAs in liver tissues from 12 HBV-related HCCs and 14 HCV-related HCCs, and 6 miRNAs were found to be markedly decreased in the HBV group and 13 miRNAs were decreased in the HCV group. Guo *et al*[14] analyzed the miRNA expression profiles of 39 cases of HBsAg positive and 11 cases of HBsAg negative liver tissues using microarrays. They showed that 9 miRNAs were up-regulated, with miR-373 being the most significantly up-regulated, and the up-regulation was associated with copy number variation of the miRs-371-3 gene cluster. The copy number variation of the miRs-371-3 gene cluster may be due to gene instability resultant from HBV integration as the genomic locus for miRs-372/373 is near to the fragile genomic site FRA19A, a known hotspot for the integration of HBV DNA[87,88]. Mizuguchi *et al*[89] analyzed the miRNA transcriptome in HBV-related HCC using combined conventional cloning with GS 454 sequencing technology. They identified more than 314000 reliable reads from HCC and more than 268000 from corresponding liver tissues for human miRNAs registered in miRBase. MiR-122, miR-21, and miR-34a were found to be expressed aberrantly in liver cancer by clone count analysis. Therefore, the combination of sequencing and bioinformatics may benefit the discovery of novel markers in HBV-related liver disease.

The identification of altered miRNA profiles in HBV-associated HCC has led to miRNA target identification and has increased our understanding of the mechanisms underlying HCC development and progression. MiRNAs can be classified as tumor suppressor-like and oncogene-like based on their functions in HCC. Furthermore, tumor suppressor-like miRNAs are often down-regulated and oncogene-like miRNAs are often up-regulated in HCC. To date, most of the miRNAs discovered in HBV-associated HCC belong to the tumor suppressor-like class. Ji *et al*[90] examined miRNA expression patterns, survival and response to IFN-α in a panel of 455 patients with mainly HBV-associated HCC. They found that miR-26 expression was frequently decreased in HCC patients and determined that patients with low miR-26 expression in tumors had a better response to interferon therapy but shorter survivals than patients with high miRNA-26 expression. In a study by Huang *et al*[91], miR-152 was found to be frequently down-regulated in HBV-related HCC compared to the paired non-tumor hepatic tissues and was found to be involved in the regulation of abnormal DNA methylation status. Further study showed that down-regulated miR-152 induced global DNA hypermethylation and increased the methylation levels of two tumor suppressor genes, glutathione S-transferase pi 1 (GSTP1) and E-cadherin 1 (CDH1), by allowing expression of DNA methyltransferase 1(DNMT1). Chen *et al*[92] found that miR-129-2 expression was repressed in HBV-HCC tissues compared to the adjacent non-tumor tissues, and miR-129-2 methylation was significantly increased both in frequency and intensity in tumor tissues. Further investigation showed this methylation-mediated repression of miR-129-2 may be involved in HCC development through enhancing oncogenic SOX4 expression. Together with other reports, all of the findings suggested that epigenetic regulation, such as DNA methylation and histone modification, of miRNA expression play an important role in HCC development[93,94]. Wang *et al*[95] reported that miR-29c was significantly down-regulated in HBV-related HCC. They showed that miR-29c functioned as a tumor suppressor in HCC development by targeting tumor necrosis factor alpha-induced protein 3 (TNFAIP3), a key regulator in inflammation and immunity.

One study reported that miR-22 was overexpressed in male tumor adjacent tissues as determined by SOLiD sequencing[96]. Overexpressed miR-22 was correlated with low estrogen receptor α (ERα) and high IL-1a expression in HBV-associated patients. These results may explain the high incidence of HBV-associated HCC in the male population. Another miRNA associated with ERα is miR-18a. Elevated miR-18a was observed in female HCC tissues compared to male HCC tissues in HBV-related HCC. MiRNA-18a may promote HCC development in females by suppressing the expression of ERα[97]. In Zhang *et al*[56]'s study, they demonstrated that miR-143 was dramatically increased by HBx-mediated NF-κB activation in metastatic HBV-HCC in both p21-HBx transgenic mice and HCC patients. Additionally, miR-143 was found to promote cancer cell invasion/migration. MiR-143 behaved as an oncogene in HCC through repressing expression of fibronectin type III domain containing 3B (FNDC3B).

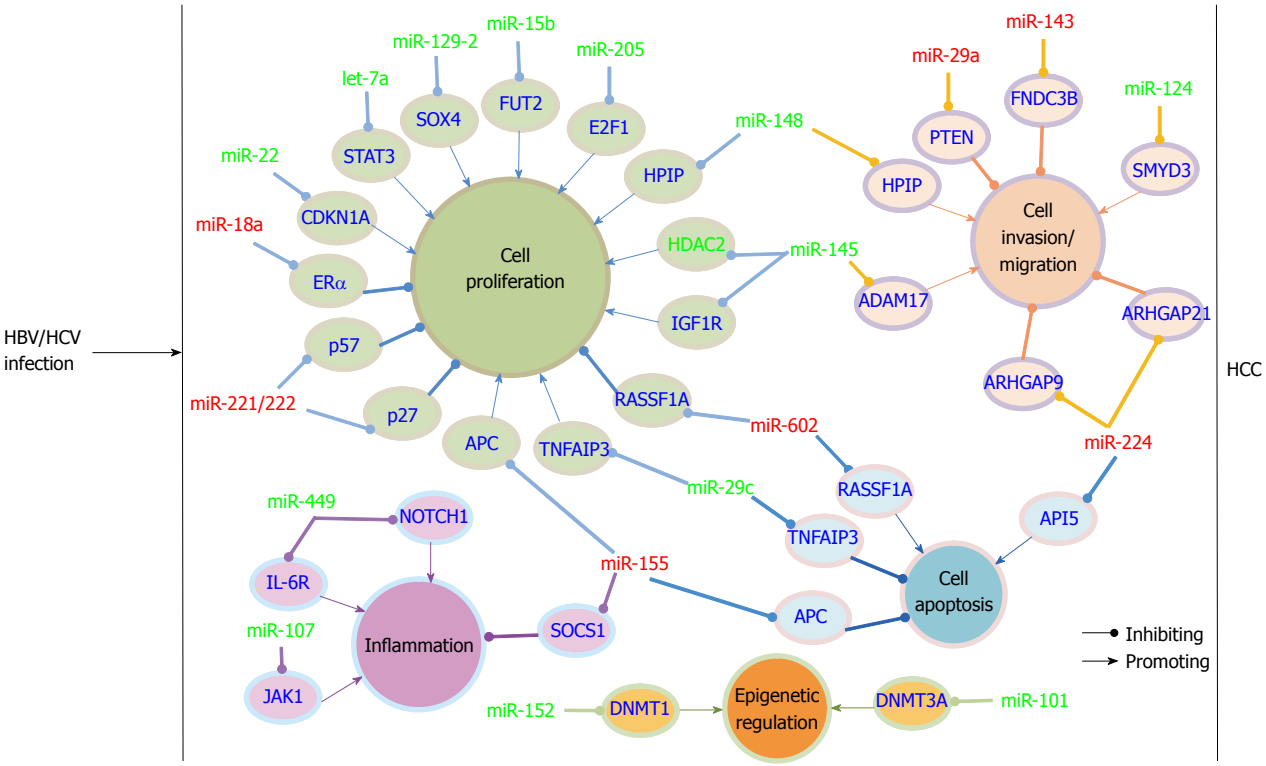

Figure 2 Summary of miRNAs and their targets associated hepatocellular carcinoma after hepatitis B virus or hepatitis C virus infection. miRNAs which are up-regulated in hepatocellular carcinoma (HCC) are indicated in red and miRNAs which are down-regulated in hepatocellular carcinoma are indicated in green. Data presented in this figure is based on literature and non-exhaustive. HBV/HCV: Hepatitis B virus or hepatitis C virus.

In a study by Yang *et al*[98], 14 miRNAs were found to be aberrantly expressed in HBV-related patients. MiR-602 showed a progressive increase trend from chronic hepatitis to HCC compared to normal livers and played a pro-carcinogenic role in HBV- associated hepatocarcinogenesis. Up-regulated miR-602 acted as an oncogene by repressing expression of tumor suppressive gene RAS association domain family 1A (RASSF1A). In a study by Gao *et al*[99], they investigated the expression of 7 cancer-related miRNAs during the early stages of HCC. Down-regulation of tumor suppressive-like miR-145 and miR-199b, and up-regulation of miR-224, was frequently observed from low grade dysplastic nodule to high grade dysplastic nodule and HCC. Moreover, in HBV-associated HCC patients, the expression of low-Atg5 (autophagy-related genes), high-miR-224 and low-Smad4 showed significant correlation with HBV infection and poor overall survival rate[100].

Recently, change in serum or plasma miRNA expression pattern was also discovered in HBV-associated patients. Li *et al*[101] examined the miRNA expression profiles in serum from 210 controls and 135 HBV-, 48 HCV-, and 120 HCC-related individuals by Solexa sequencing followed by TaqMan probe-based quantitative real-time PCR validation. They identified 13 miRNAs that were differentially expressed in HBV serum compared to control serum. Two of them, miR-375 and miR-92a, were also identified as HBV specific. This is the first report that serum miRNA profiles can serve as a biomarker for HBV infection. Later, additional miRNAs were found to be significantly deregulated in serum or plasma from patients with chronic HBV infection (CHB), HBV-associated HCC or occult HBV infection compared with healthy controls, which suggests that serum or plasma miRNAs have the potential to serve as biomarkers in detecting HBV-specific cases[102-111]. Interestingly, a recent study reported that serum HBsAg particles carried selective pools of hepatocellular miRNAs and may play a role in viral persistence, which enriched the knowledge of HBV-host interaction[112] (Figure 2).

MiRNA expression profile after HCV infection and their functions

Different HCV genotypes induced different miRNA expression changes in hepatoma cells. Liu *et al*[113] compared the miRNA expression profile between Huh7.5.1 hepatoma cells infected with HCV JFH-1 virus and the same cell type infected with UV-inactivated HCV virus with miRNA microarray analysis. One-hundred eight miRNAs were differentially expressed (greater than 2-fold change) at 4 d post infection. Most (60%) were up-regulated in HCV-infected cells, and some of them were identified to be involved in HCV entry, replication and propagation. In Huh7 cells, miR-192/miR-215 and miR-194 were up-regulated and miR-320 and miR-491 were down-regulated after HCV infection. Moreover, overexpression of miR-192/miR-215 and miR-491 *in vitro* significantly increased HCV replicon abundance[41]. In Huh7.5 cells, 7 miRNAs (miR-30b, miR-30c, miR-130a, miR-192, miR-301, miR-324-5p, and miR-565), which were linked to genes associated with HCV entry and replication, were

down-regulated after infection with HCV genotype 2a[114]. The miR-192 expression is inconsistent in the two studies. This may be due to the difference in cell type, HCV genotype and sampling time after HCV infection. Steuerwald *et al*[115] found that 43 miRNAs were differently expressed between Con1 cells containing a full-length HCV genotype 1b replicon and Huh 7.5 parental hepatoma cells. In another hepatoma cell line, HepG2, stably transfected with full length HCV genotype 1b, 75 miRNAs were found to be aberrantly expressed. The miRNAs miR-193b, miR-768-3p, and miR-585 were the most significantly up-regulated. Up-regulated miR-193b made the malignant hepatocytes more sensitive to sorafenib by modulation of the anti-apoptotic protein Mcl-1. The miRNAs that had been reported to be regulated by the HCV core protein or non-structure proteins (NS3/4A, NS4B and NS5A) included miR-93, miR-345, miR-124, miR-21, and miR-27[116-120].

Varnholt *et al*[121] examined the miRNA expression profiles in HCV-infected patients with premalignant dysplastic liver nodules and hepatocellular carcinomas by real-time PCR. Ten miRNAs were more than 2-fold overexpressed, and 19 were less than 0.4-fold underexpressed. Among the overexpressed miRNAs, miR-122, miR-100, and miR-10a were consistently overexpressed across all liver nodules. MiR-198 and miR-145 were underexpressed progressively from low-grade dysplastic nodule to high-grade dysplastic nodule to HCC and further to kidney metastasis. Marquez *et al*[122] found that miR-122 and miR-21 expression were altered in HCV-infected liver. The miR-21 levels were correlated, while miR-122 levels were inversely correlated, with viral load, fibrosis and serum liver transaminase levels. Zhang *et al*[123] found that miR-155 was significantly increased by HCV-RNA-induced NF-κB activation in chronic HCV-infected patients and HCV-HCC compared to healthy controls. Up-regulated miR-155 repressed Adenomatous polyposis coli (APC) and subsequently activated Wnt signaling, which resulted in hepatocyte proliferation and tumorigenesis. P65/NF-κB activated miR-224 expression in both peri-tumoral cirrhotic livers and HCC samples from HCV-infected patients as determined by real-time PCR. MiR-224 promoted HCC cell migration and invasion, which is a key determinant in HCC development and progression[124]. A genome-wide microarray analysis performed by Sarma *et al*[125] showed that a distinct miRNA expression profile existed in HCV-infected liver tissue compared to the normal liver. Particularly, miR-449a was specifically down-regulated in HCV-infected patients compared to the other three groups, and down-regulated miR-449a promoted inflammatory marker YKL40 expression by modulating the NOTCH signaling pathway. Diaz *et al*[126] examined the expression of 2226 miRNAs in patients with HCV-associated HCC, HCC-associated non-tumorous cirrhosis, HCV-associated cirrhosis without HCC, HBV-associated ALF and normal liver tissue using microarrays. They found 18 miRNAs that were specifically expressed in HCV-associated HCC and were connected in a regulatory network, including p53 tumor suppressor, the PTEN phosphatase and retinoic acid signaling.

Ogawa *et al*[127] found that the miR-221/222 levels increased with the progression of liver fibrosis in HCV-infected patients and significantly correlated with the expression of a1 (I) collagen (Col1A1) and α-smooth muscle actin (αSMA). MiR-221/222 was the first miRNA that was reported to be related to liver fibrosis and may be useful in the prediction of liver fibrosis progression. Ramachandran *et al*[128] found that several miRNAs expression were altered by chronic HCV infection by using miRNA expression profiling. HCV-induced-miR-200c promotes hepatic fibrosis by repressing the expression of FAS associated phosphatase 1 (FAP-1), a critical regulator of Src and MAP kinase pathway. It was reported that miRNA-107 and miRNA-449a were decreased following HCV infection in patients with HCV-mediated liver diseases. Further function analysis showed that they regulate CCL2 expression by activation of the IL-6-mediated signaling cascade, which may be involved in HCV-mediated induction of inflammatory responses and fibrosis[129]. A report showed that 5 miRNAs were significantly overexpressed and 7 miRNAs were markedly reduced in splenic marginal zone lymphoma (SMZL) from HCV-positive patients[130]. MiR-26b, a known tumor suppressor, was found to be significantly reduced in SMZL. This result suggested that HCV may contribute to lymphoma development. With the hypothesis that miRNA expression profiles from liver grafts could distinguish the severity of HCV recurrence and discriminate this from acute cellular rejection (ACR), Joshi *et al*[131] found that miR-146a, miR-19a, miR-20a, and let-7e were overexpressed in patients with slow HCV fibrosis progression compared to patients with fast HCV fibrosis progression after liver transplantation. Distinct miRNA profiles exist between patients with HCV recurrence and ACR. Moreover, altered hepatic expression of miRNA is associated with HCV recurrence and antiviral therapy in liver transplant recipients for hepatitis C virus cirrhosis[132].

In chronic HCV-infected patients, serum miR-122 was increased, and the levels of miR-122 were correlated with serum ALT but not with HCV RNA[133]. However, a later study showed that miR-122 may just mirror acute liver injury with the finding that the levels of miR-122 that were high in the early stage of fibrosis were reduced in advanced fibrosis stages during chronic HCV infection[134]. However, Cermelli *et al*[135] found that serum levels of miR-122, miR-34a, and miR-16 in chronic HCV-infected patients were steadily increased during the course of HCV infection, and miR-122 and miR-34a levels were positively correlated with disease severity. The discordant change trend of miR-122 may be due to the lack of a consistent internal control for circular miRNA measurements or due to the small patient population. Moreover, the serum miR-122 kinetics could predict the treatment results, as patients with higher serum miR-122 showed early and sustained virologic response to pegylated IFN plus ribavirin therapy[38]. In addition to miRNA-122, other miRNAs were also found to be significantly de-

regulated in serum from HCV-related patients and were associated with either HCV infection or HCV-mediated liver disease progress[136-140]. MiRNA deregulation was also found in peripheral blood mononuclear cells (PBMCs) of HCV-infected patients. Deregulation of miRNAs may play a key role in HCV-related pathogenesis[141,142]. One study showed that 27 miRNAs and 476 mRNAs were differentially expressed in PBMCs from HCV/HIV co-infected patients when compared to controls by performing genome-wide mRNA and miRNA analysis, this is the first report of miRNAs specific for HCV/HIV co-infection[143]. Abdalla *et al*[144] found that miR-323, miR-449, miR-502d, miR-92b, miR-516-5p, and miR-650 were down-regulated while miR-335, miR-618, miR-625, miR-532, and miR-7 were up-regulated in urine from both the HCC-post HCV-positive group and the HCV-positive group compared to the control group. Further study showed that the aberrant expression of miR-618 and miR-650 can be used as a marker for the early diagnosis of HCC from HCV-positive patients (Figure 2).

MIRNAS AS POTENTIAL DIAGNOSTIC OR PROGNOSTIC MARKERS OR THERAPEUTIC TARGETS IN HCC AND HEPATITIS B/C INFECTION

It has been shown that miRNAs play important roles in HBV/HCV infection and the associated liver disease. Moreover, differential expression patterns of miRNAs have been observed in the livers or serum of HBV- and HCV-infected patients. The unique miRNA expression profile in HBV- or HCV-related diseases suggested that they may represent novel biomarkers for diagnosis or prognosis or can be exploited as therapeutic agents or targets in the treatment and prevention of these diseases.

MiRNA as diagnostic markers in HBV- or HCV-related diseases

In HBV-related liver diseases, some miRNAs were aberrantly expressed in liver tissues, such as miR-148a, miR-96, miR-29c, and miR-602, were related to virus infection and disease progression[81,84,95,98]. Therefore, miRNAs may serve as diagnostic markers. For example, miR-602 was overexpressed from the very early stage of chronic hepatitis to HBV-positive cirrhosis and HCC[98]. MiR-602 played an important regulatory role in HBV-mediated hepatocarcinogenesis by inhibiting the tumor suppressive gene RASSF1A. Therefore, this miRNA can serve as an early diagnostic marker for HBV-mediated HCC. In HCV-related liver diseases, some deregulated miRNAs in liver tissues also have diagnostic potential. HCV-induced miR-155 could promote hepatocyte proliferation and tumorigenesis by activating Wnt signaling and thus may be a useful biomarker for early diagnostics of HCV-HCC[123]. With high specificity and selectivity, 18 miRNAs exclusively expressed in HCV-HCC were identified by Diaz *et al*[126] and displayed particular clinical value in diagnosing HCV-HCC. Up-regulated miR-199a, miR-199a*, miR-200a, miR-200b, and miR-221/222 were significantly correlated to the grade of liver fibrosis and may serve as biomarkers for stellate cell activation and liver fibrosis progression[127].

Although the miRNA expression profiles in liver tissues were helpful in discriminating HCC from non-tumor tissues, they are not ideal as markers in the early diagnosis of HCC for their invasiveness. Circulating miRNAs were specific and stable in body fluids such as blood or urine and can be used as potential diagnostic markers not only for HBV or HCV infection but also for the development of HCC. In separating the control and HBV groups, serum miR-375 or miR-10a individually had a 97.5% area under curve (AUC) of receiver operating characteristic (ROC) while the combination of miR-375, miR-10a, miR-223, and miR-423 resulted in 100% AUC. Serum miRNAs including miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p could clearly separate the HBV-positive HCC group from the control with an AUC of 99.9%[101]. A plasma miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) had a high diagnostic accuracy of HBV-positive HCC regardless of disease status (Barcelona Clinic Liver Cancer stages 0, A, B, and C). This miRNA panel could also discriminate HCC from healthy liver, chronic hepatitis B and cirrhosis[108]. The expression profiles of miR-21, miR-122, and miR-223 could serve as a novel biomarker for liver injury but not specifically for detection of HCC in chronic HBV-infected patients[105]. The serum miR-210 level may serve as a molecular biomarker for the severity of hepatitis in patients with hepatitis B[110]. The serum miR-125b-5p and miR223-3p are potentially very early diagnostic biomarkers of HBV-related HCC even at chronic hepatitis B stage of liver disease[111]. Four serum miRNA signatures (let-7c, miR-23b, miR-122, and miR-150) can clearly separate occult HBV-infected patients from the control group with an AUC of 99.9%, although larger population studies are required to validate these findings[107]. Plasma miR-20a and miR-92a were increased in acute and chronic HCV-infected patients and HCV-infected fibrosis patients and thus may serve as biomarkers for the early detection of HCV infection[137]. The urinary miRNAs signatures found by Abdalla *et al*[144] is of great value for the early diagnosis of HCC, before the onset of disease in HCV-positive patients (Table 1).

MiRNAs as prognostic markers in HBV- or HCV-related diseases

MiRNAs were also suitable for predicting the HCC clinical outcomes. A set of 19 miRNAs were significantly altered in cirrhotic and hepatitis B or C virus-positive livers, and this miRNA expression signature significantly correlated with disease outcome[83]. The expression levels of miR-26 were reduced in HCC compared with adjacent non-tumor tissues. Moreover, tumor tissue with low miRNA-26 expression was associated with shorter overall survival but better response to IFN therapy than

Table 1 miRNAs as potential hepatocellular carcinoma diagnostic and prognostic markers

miRNA	Expression (*vs* control)	HCC etiology	Clinical relevance	Ref.
Liver miRNAs				
miR-29c	Down	HBV	Diagnostic marker therapeutic agent	[95]
miR-96	Up	HBV	Diagnostic marker	[84]
miR-148	Up	HBV	Early diagnostic marker and/or therapeutic target for HBx-mediated HCC	[81]
miR-155	Up	HCV	Diagnostic marker therapeutic target	[123]
miR-221/222	Up	HCV	New markers for stellate cell activation and liver fibrosis progression	[127]
miR-602	Up	HBV	Early diagnostic marker	[98]
Serum miRNAs				
miR-18b	Up	Mainly HCV	Prognostic marker	[146]
miR-20a	Up	HCV	Sensitive biomarker for early detection of HCV infection	[137]
miR-26	Down	mainly HBV	Prognostic marker	[90]
miR-29a-5p	Up	HBV	Novel predictor for early recurrence of HBV-HCC after surgical resection	[145]
miR-92a	Up	HBV	Novel biomarker for HBV infection and HBV-HCC	[101]
miR-122	Up	HBV	Novel biomarker for liver injury but not specifically for HCC	[102,105]
miR-125b-5p	Up	HBV	Novel biomarkers of HCC in very early, even at CHB stage of liver disease	[111]
miR-210	Up	HBV	A molecular biomarker for the severity of hepatitis	[110]
miR-223	Down	HBV	Novel biomarkers of HCC in very early, even at CHB stage of liver disease	[111]
miR-224	Up	HBV	Autophagy-miR-224-Smad4 in combination could be used prognostic marker	[100]
miR-375	Up	HBV	Novel biomarker for HBV infection and HBV-HCC	[101]
miR-572	Up	HBV	Biomarker for liver injury	[102]
miR-575	Up	HBV	Biomarker for liver injury	[102]
miR-638	Up	HBV	Biomarker for liver injury	[102]
miR-744	Down	HBV	Biomarker for liver injury	[102]
Urinary miRNAs				
miR-618	Up	HCV	Early diagnostic marker of HCC among high-risk HCV patients	[144]
miR-650	Down	HCV	Early diagnostic marker of HCC among high-risk HCV patients	[144]

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

tumor tissue with high miR-26. It hence appears to be a predictor of survival[90]. MiR-29a-5p can be a novel predictor for early recurrence of HBV-related HCC after HCC resection, especially in BCLC 0/A stage HCCs[145]. In HBV-associated HCC patients, the expression of low-Atg5 (autophagy-related genes), high-miR-224 and low-Smad4 shows significant correlation with HBV infection and poor overall survival rate[100]. ROC curve analysis revealed that the levels of miR-122, miR-572, miR-575, miR-638, and miR-744 in serum can serve as molecular markers to predict liver injury resulted from chronic hepatitis B[102]. Murakami *et al*[146] found that HCC patients (mainly HCV-HCC) with high miR-18b expression had a significantly shorter relapse-free period than those with low expression after surgical resection. Joshi *et al*[131] identified a panel of miRNAs associated with the severity of HCV recurrence which can be used as biomarkers that are predictive of aggressive recurrence after liver transplantation. Similarly, Gehrau *et al*[147] identified a 9 miRNA signature that can predict the severity of hepatitis C virus recurrence after liver transplantation (Table 1).

MiRNAs as therapeutic targets or drugs in HBV- or HCV-related diseases

Many *in vitro* studies have indicated that miRNA-based therapy could efficiently inhibit HBV/HCV production and impair proliferation and invasion of the HCC cells. With the advantage that one miRNA can simultaneously target multiple genes and pathways, and one gene or pathway can be targeted by multiple miRNAs, targeting miRNA may be an efficient antiviral or anti-HCC strategy and be helpful in reducing the incidence of therapy resistance. The application of miRNA often involves two strategies: restoration of miRNA with antiviral or tumor suppressor functions by reintroducing miRNA mimics or expression from transfected/transduced vectors and the inhibition of miRNAs with virus promoting or oncogene-like functions by using miRNA inhibitors, such as antagomiRs and antimiRs. At present, only one miRNA-based drug has entered and passed through human clinical 1 and clinical 2a trials, the miRNA-122 inhibitor miravirsen. Miravirsen showed a promising antiviral activity with no obvious evidence of virus escape in treatment-naive HCV patients and thus may be useful in preventing HCV-related HCC[33,34].

Some studies also showed that miRNAs were involved in the response/resistance of HCC cells to chemotherapy[38,39,116,148-153]. The classical miRNA is miRNA-122, which accounts for more than 70% of all miRNAs present in hepatic cells. During chronic HCV infection, patients with decreased pretreatment liver miRNA-122 levels responded poorly to IFN therapy[128]. Similarly, patients with higher pretreatment serum miRNA-122 levels showed early and sustained virologic response to pegylated IFN plus ribavirin therapy[38]. Restoration of miRNA-122-sensitized HCC cells to doxorubicin, sorafenib, adriamycin and vincristine occurs through inactivation of the CDK4-PSMD10-UPR pathway or down-regulation of multidrug resistance-related genes[149-152]. Comprehen-

sive expression profiling of miRNAs in DOX-resistant and parental HCC HepG2 cells were recently analyzed by deep sequencing[153]. The authors identified a panel of differentially expressed known and novel miRNAs, and their target genes were mainly involved in the MAPK signaling pathway by function annotation analysis, which may help us to overcome DOX resistance in future HCC chemotherapy.

CONCLUSION

The complex interactions between miRNA and HBV/HCV were discussed in this review. HBV/HCV infection elicited changes in the host miRNA expression profile, and the altered miRNAs could in turn facilitate the virus life cycle and the development of virus-associated diseases, which suggested that the deregulated miRNA profiles may serve as biomarkers and therapy targets for virus-mediated diseases. For their stability and specificity of expression in tissue and circulation, miRNAs are good biomarkers for early diagnosis and prognostic prediction. But the patient population, the sampling and testing technologies are often different in different studies, and the present testing technologies (including microarray, real time-PCR, and next generation sequencing) are either high cost or low sensitivity, all these factors limit their clinical application. With the broad regulatory roles of miRNAs, and for their small size and less antigenicity, miRNAs are good candidates for therapeutic targeting. However, in addition to the side effects and high cost, the stability of miRNA modulators and the specific delivery system are the major challenges to overcome in advancing miRNA-based strategies from bench to bedside. In view of the advantages and limitations of application of miRNA in clinic, further research into the precise molecular mechanism by which miRNAs regulate the pathogenesis of virus-associated diseases will be helpful in early diagnosis, prognostic prediction and effective treatment of these diseases.

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REVIEW

Recent insights into farnesoid X receptor in non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and is one of the most prevalent liver disorders worldwide. NAFLD can gradually progress to liver inflammation, fibrosis, cirrhosis and even hepatocellular carcinoma. However, the pathogenesis of NAFLD is complex, and no efficient pharmaceutic treatments have yet been established for NAFLD. Accumulating data have shown that the farnesoid X receptor (FXR) plays important roles not only in bile acid metabolism, but also in lipid and carbohydrate homeostasis, inflammatory responses, among others. In this review, we aim to highlight the role of FXR in the pathogenesis and treatment of NAFLD.

Key words: Farnesoid X receptor; Non-alcoholic fatty liver disease; Mechanism; Therapy; Lipid metabolism

Core tip: Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent liver disorders worldwide and has great risk potentials. While the mechanisms under NAFLD are still in the mist, farnesoid X receptor (FXR) provides a new aspect in this field. In addition to regulate bile acid metabolism, FXR can also be actively involved in lipid (cholesterol, triglyceride, fatty acid) and glucose metabolism, furthermore, FXR participates in regulating inflammation and NAFLD progression. Several FXR agonists are identified and both experimentally and clinically proved to be optimistic in preventing and treating NAFLD, indicating FXR quite a therapeutic target for NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by the presence of lipid droplets in hepatocytes in the absence of alcohol consumption. The spectrum of NAFLD is from simple steatosis to non-alcoholic steatohepatitis (NASH) and eventually cirrhosis and hepatocellular carcinoma (HCC). NAFLD is affecting 15%-40% of the general population[1], and among them at least 10%-20% would develop to NASH[2], which is a potentially serious condition with poor prognosis. NASH is currently the most rapidly growing indication for liver transplantation (LT) in patients with HCC in the United States, and is predicted to become the leading indication for LT in the near future[3]. A recent large cohort study indicated that the prevalence of colorectal malignant neoplasm is also closely associated with NAFLD[4].

Frequently, NAFLD clusters with metabolic abnormalities, including type 2 diabetes, obesity, hypertension, hyperlipidemia, *etc*. Growing evidence has suggested that NAFLD is associated not only with liver-related mortality and morbidity but also with an increased incidence of chronic kidney disease[5,6], cardiovascular disease[5,7] and aortic valve sclerosis[8]. NAFLD is thus becoming a major health issue. To date, no optimal treatment has been found, underscoring the need for further efforts in elucidating the pathogenesis of NAFLD and distinguishing effective pharmacological therapies.

Farnesoid X receptor (FXR) is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily, it is abundantly expressed in the liver, intestine, kidney, and adrenal cortex, while low levels of FXR have been detected in a variety of tissues including the heart, lung, adipose tissue, *etc*[9,10]. It was initially thought to be the receptor of intermediate metabolites, farnesol, from which the name "Farnesoid X receptor" was derived. In 1999, bile acids (BAs) were found as the natural ligands of FXR, which has since been known as bile acid receptor[11]. As a transcription factor, it binds to DNA either as a monomer or as a heterodimer with a common partner-retinoid X receptor (RXR) to regulate the expression of various genes involved in BA, lipid and glucose metabolism[12,13].

It has been observed that hepatic expression of FXR is decreased in NAFLD patients, which is associated with hepatic triglyceride (TG) accumulation and hepatic steatosis[14], FXR deficiency animal models display hepatic steatosis, hyperlipidaemia, hyperglycemia, BA overload, inflammation and fibrosis[15-18]. However, these can be improved by FXR activation[19,20], indicating FXR could be a key regulator of metabolic homeostasis. Thus FXR appears to be a promising target for the treatment of NAFLD.

POTENTIAL PATHOGENESIS OF NAFLD

The pathogenesis and progression of NAFLD are multifactorial and not quite so clear, while generally explained by the "two-hit" theory[21]. The "first hit" is hepatic fat accumulation owing to increased hepatic *de novo* lipogenesis (DNL) and fatty acid uptake, inhibition of fatty acid β oxidation (FAO), impaired TG clearance and decreased very-low-density lipoprotein (VLDL) export[22]. Oxidative stress and subsequent inflammation are key factors of the "second hit", which ultimately cause further liver damage. Studies have shown that multiple parallel hits, including genetic differences, intestinal microbiota, adipose-derived cytokines and so on account for the progression of NAFLD[23].

Loss of the body's ability to retain excess lipids in "classical" adipose tissue stores can lead to the overdevelopment of ectopic fat deposition, often creating severe perturbations of both glucose and lipid homeostasis[24]. Excessive fat accumulation in the liver is recognized as a pathological state. Hepatic ectopic fat deposition, especially TG, cholesterol and fatty acid eventually lead to disordered hepatic lipid metabolism.

TG derives from the esterification of free fatty acid (FFA) that may come from dietary fats, adipose tissue and DNL, and can be used for energy through FAO in mitochondria. Hepatic TG lipolysis is mediated by lipases, which release FFA for oxidation. After synthesis, hepatic TG may be stored as lipid droplets or packaged with ApoB into VLDL and then secreted into circulation[25].

MECHANISMS OF FXR IN NAFLD

Although inappropriate lipid metabolism, insulin resistance, and inflammation represent important risk factors for the development of NAFLD, the precise mechanisms controlling disease pathogenesis remain largely undefined. Recent studies on FXR have provided new opportunities to elucidate the pathogenesis of NAFLD, and the beneficial role of FXR on NAFLD is through multiple mechanisms.

FXR in regulating bile acid metabolism

BAs are the end products of cholesterol catabolism, produced in the liver, then secreted into the bile canaliculi and subsequently stored in the gall bladder. After ingestion of food, bile flows into the duodenum, where it contributes to the absorption of dietary lipid and fat-soluble vitamins. Most of these BAs (95%) are then reabsorbed from the terminal ileum and transported back to the liver *via* the portal vein, which is known as enterohepatic circulation. Only about 5% of them escapes from reabsorption per cycle and expels from the body in the feces[9,10,26]. BA synthesis *via* two different pathways: the classical pathway and alternative pathway. Two primary BAs cholic acid (CA), chenodeoxycholic acid (CDCA) are the end products of these two pathways. Secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) are derived from primary BAs in the intestine by bacterial enzymes.

Three enzymes play major regulatory roles in these two pathways. Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the classical pathway, whereas sterol-27 hydroxylase (CYP27A1) is the first enzyme in the alternative pathway, followed by sterol 12 α-hydroxylase (CYP8B1)[10,26]. Several members participate in bile acid transport and enterohepatic circulation. The bile salt export pump (BSEP) is mainly responsible for bile acid transport at the canalicular membrane. Na^+-dependent taurocholate transporter (NTCP) is responsible for basolateral bile acid transport into the hepatocytes. BAs are reabsorbed mostly in the terminal ileum, and are mainly mediated by the apical sodium-dependent bile salt transporter (ASBT). Once absorbed into the enterocytes, BAs then bind the intestinal bile acid binding protein (I-BABP) and are transported to the basolateral membrane for secretion[27].

Although BAs have many physiological roles, abnormal high levels of BAs would increase the risk of hepatotoxicity, because they can cause oxidative stress,

inflammation, necrosis, and eventually fibrosis and cirrhosis[28,29], which are key roles of the pathogenesis of NAFLD. On the other hand, they also function as signaling molecules and metabolic regulators that activate dedicated BA receptors such as FXR to protect against toxic accumulation of BAs, and regulate hepatic lipid, glucose, and energy homeostasis and maintain metabolic homeostasis[10,30].

FXR plays a central role in bile acid homeostasis by regulating genes involved in bile acid synthesis, secretion and reabsorption. FXR inhibits *de novo* BA biosynthesis through up-regulation of the small heterodimer partner (SHP), which interacts with and represses the transcriptional activator, liver related homolog 1 (LRH-1) and hepatocyte nuclear factor-4α (HNF-4α), thus bind to the *CYP7A1* gene promoter, and inhibiting *CYP7A1* gene transcription[31,32]. Additionally, FXR can induce intestinal fibroblast growth factor 19 (FGF19) in humans, as well as FGF15, the mouse ortholog of human FGF19, which then activate the cell-surface receptor, FGF receptor 4 (FGFR4), to eventually inhibit *CYP7A1* gene transcription and bile acid synthesis intracellular *via* intracellular Jun N-terminal kinase (JNK) pathway[33-36]. FXR encompasses the regulation of the enterohepatic circulation. Through up-regulation of BSEP and multidrug resistance protein 2 (MRP2, human canalicular bilirubin conjugate export pump) and inhibition of NTCP, FXR reduces hepatocellular BA levels by stimulating bile acid secretion at the canalicular membrane and limit bile acid uptake from the portal circulation[37-39]. FXR is also able to induce alternative basolateral BA transport through organic solute transporter α/β (OSTα/β), to efflux BAs to systemic circulation and, subsequently, are eliminated by renal excretion[38,40]. Given the above, FXR regulates the synthesis and export of BAs, hence activation of FXR can protect against the liver from toxic accumulation of BAs.

FXR on cholesterol metabolism

In recent years, the role of FXR in cholesterol metabolism has been widely explored. Emerging experimental and clinical evidence has linked altered hepatic cholesterol homeostasis and free cholesterol (FC) accumulation to the risk and severity of NAFLD and the pathogenesis of NASH[41]. It is considered that hepatic accumulation of cholesterol rather than TG may play a critical role in the NAFLD progression[42].

In hepatocytes, cholesterol homeostasis pathways include cholesterol *de novo* synthesis, uptake in the form of low density lipoprotein (LDL) and chylomicron remnants, excretion into the blood in the form of VLDL, excretion and uptake through bile, and synthesis of BAs and their excretion[42]. Since FXR is a key regulator of bile acid metabolism, it is also critical in maintaining cholesterol homeostasis. FXR deficiency mice display increased levels of hepatic and serum cholesterol[43,44], and FXR negatively regulates cholesterol levels *via* various mechanisms.

LDL receptor (LDLR), the scavenger receptor class B type I (SR-BI) and cluster differentiation protein-36 (CD-36) are involved in hepatic cholesterol uptake. Increased LDLR and CD-36 expression, and decreased SR-BI expression are detected in NAFLD, which correlates with the severity of steatosis[45]. Activation of FXR represses the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9), an inhibitor of LDLR, thus increases LDLR activity, and potentiates the hypolipidemic effect of statins[46]. SR-BI is critical for reverse cholesterol transport by transporting high-density lipoprotein (HDL) cholesterol into liver where a part of the cholesterol is metabolized to BAs[47,48]. FXR null mice exhibit reduced SR-BI expression[44]. A recent study showed that FXR positively regulates SR-BI expression, and three binding sites in the first intron of the *SR-BI* gene were identified[47]. Meanwhile, FXR induced reduction of CD36 also effectively prevents liver from steatosis[49]. In the liver, FXR enhances ATP-binding cassette G member 5 and member 8 (ABCG5/G8) expression, a heterodimeric cholesterol efflux transporter, which accounts for increased cholesterol excretion[50]. Collectively, FXR inhibits cholesterol uptake and synthesis and promotes cholesterol excretion, eventually improves cholesterol overload.

FXR in mediating fatty acid and triglyceride metabolism

Hepatic steatosis is the hallmark of NAFLD due to an imbalance between TG synthesis and clearance. From a liver centric point of view, this imbalance results from abnormalities in one or more of the following four processes: (1) hepatic uptake of fatty acid, lipoprotein and glucose; (2) *de novo* TG synthesis; (3) TG degradation and FAO; and (4) lipoprotein secretion in the form of VLDL[51].

FXR has shown considerable impact on lipogenesis. Hepatic lipogenesis is mainly regulated by sterol regulatory element binding protein 1c (SREBP-1c), which is known as the master regulator of lipid biosynthesis and regulates the expression of several genes involved in lipogenesis[52]. FXR activation can inhibit the expression of SREBP-1c and its target enzymes, such as fatty acid synthase (FAS), stearoyl-coenzyme A desaturase 1 (SCD-1) and acetyl-CoA carboxylase (ACC), and prevent excessive fatty acid synthesis and overproduction of TG[19,53,54]. FXR null mice develop hepatic steatosis and hypertriglyceridemia[55]. In NAFLD patients, decreased expression of hepatic FXR also displays elevated TG synthesis, due to increased expression of SREBP-1c[14]. FXR activation effectively prevents hepatic TG accumulation; the underlying mechanisms may be due to FXR-mediated SHP activation, thus suppressing the expression of SREBP-1c and its lipogenic target genes[18]. Other mechanisms independent of the FXR-SHP-SREBP-1c pathway may also contribute to FXR-mediated TG homeostasis[25].

FXR also demonstrates an ability to enhance TG clearance. FXR is known to induce apolipoprotein C-II (Apo C-II) and apolipoprotein AIV (Apo AIV) and inhibit apolipoprotein C-III (Apo C-III) and angiopoietin-like 3 expression, thus activating lipoprotein lipase (LPL)-

mediated lipolysis of TG rich lipoproteins[56]. Peroxisome proliferator-activated receptor alpha (PPARα) is a key regulator of FAO and activation of FXR induces the expression of PPARα and its target gene, carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme in FAO[57]. Furthermore, FXR activation by natural and synthetic BAs increases the expression and secretion of fibroblast growth factor 21 (FGF21), which has been reported to profoundly reduce hepatic TG levels *via* inhibition of SREBP-1c[58,59]. Furthermore, FGF21 induces gluconeogenesis, FAO, and ketogenesis in the liver[60].

In addition, FXR-induced hepatic expression of aldo-keto reductase B7 (Akr1b7) has revealed a striking effect on ameliorating hepatic lipid accumulation in *db/db* mice[61]. A recent study showed that hepatic carboxylesterase 1 (CES1) plays a key role in regulating both normal and FXR-controlled lipid homeostasis. Overexpression of hepatic CES1 lowered hepatic TG, while knockdown of hepatic CES1 increased hepatic TG and plasma cholesterol levels. These effects likely resulted from the TG hydrolase activity of CES1. Activation of FXR induced hepatic CES1, and reduced the levels of hepatic and plasma TG as well as plasma cholesterol in a CES1-dependent manner[62]. Lu *et al*[63] have identified YY1 as a novel transcription factor involved in hepatic TG metabolism in obesity. YY1 expression is markedly up-regulated in HFD-induced obese mice and NAFLD patients. YY1 suppresses FXR expression *via* interaction with the YY1 binding site at the first intron of the *FXR* gene. Liver-specific ablation of YY1 ameliorates liver TG accumulation in obese mice.

FXR and inflammation

Inflammation and fibrosis are main pathological manifestations of NASH. Recently, it has become clear that FXR can down-regulate genes involved in inflammation. FXR deficiency is considered as a significant risk factor in the development of NASH. $LDLR^{-/-}/FXR^{-/-}$ mice fed a high-fat diet (HFD) display higher levels of pro-inflammatory and pro-fibrogenic cytokines, such as tumor necrosis factor α (TNFα), intercellular adhesion molecule-1 (ICAM-1), α-smooth muscle actin (α-SMA), tissue inhibitor of metalloproteinase (TIMP)-1, transforming growth factor (TGFβ), procollagen 1α1 and type 1 collagen compared to $LDLR^{-/-}/FXR^{+/+}$ mice[16]. These studies indicated that activation of FXR may be a therapeutic target in curing NASH.

Indeed, FXR activation appears to protect mice against methionine and choline-deficient (MCD) diet-induced NASH. The reduction in inflammatory cell infiltration and hepatic fibrosis correlated with decreased levels of hepatic inflammation markers such as keratinocyte derived chemokine (mKC), MCP-1, VCAM-1, *etc*, and fibrosis markers such as TIMP-1, α1(I) collagen, α-SMA, TGF-β1, matrix metalloproteinase 2 (MMP-2) and α2(I) collagen[20]. Furthermore, the observation that FXR null mice are more susceptible to LPS-induced liver injury, indicating a direct anti-inflammatory role of FXR, which has been explained *via* negatively mediating the nuclear factor kappa-B (NF-κB) pathway[64]. Additionally, in the intestine, FXR is required to improve biliary obstruction, inhibit bacterial overgrowth, mucosal injury and bacterial translocation[65]. Another anti-inflammatory effect of FXR involves induction of suppressor of cytokine signaling 3 (SOCS3) that inhibits signal transducer and activator of transcription (STAT3) signaling[66]. Recently, Peng *et al*[67] identified *REC*K, a membrane-anchored inhibitor of *MMP-9*, as a novel target gene of FXR in mouse liver. Whether the FXR agonist attenuates hepatic inflammation and fibrosis in a mouse NASH model through the FXR-RECK-MMP-9 cascade still needs further investigation. On the other hand, cholesterol over-intake and BAs accumulation are correlated with the onset and severity in NASH, while the role of FXR in the process need to be further clarified[68].

Some microRNAs have been found to be target genes of FXR, and regulate the process of liver fibrogenesis. FXR-mediated miR-29a up-regulation in hepatic stellate cells (HSCs) leads to decreased amounts of extracellular matrix, and thus protects against liver fibrosis[69]. In another study, liver tissues from patients with severe fibrosis are found to have lower levels of FXR and liver kinase B1 (LKB1) with up-regulated miR-199a-3p. FXR is further confirmed to protect hepatocytes from injury by repressing miR-199a-3p and thereby increasing levels of LKB1[70]. Taken together, the anti-inflammatory actions of FXR are obtained from intra-hepatic and extra-hepatic mechanisms, more experiments are needed to elucidate the molecular mechanisms under the actions.

Other possible mechanisms

Type 2 diabetes is an established risk factor for development of hepatic steatosis and NAFLD. Indeed, the prevalence of NAFLD is higher in patients with type 2 diabetes[71]. Several animal studies have shown that FXR activation can improve insulin sensitivity and down-regulate phosphoenoylpyruvate kinase (PEPCK) and glucose-6-phosphatase (G-6-Pase), two key enzymes in gluconeogenesis[17,49]. Activation of FXR is also reported to induce the phosphorylation of glycogen synthase kinase 3β (GSK3β) to enhance glycogen storage in *db/db* mice[72]. FXR also has a novel role in promoting liver regeneration/repair after liver damage, including physical resection or toxic injury[73]. Apart from this, research has addressed the role of FXR on oxidative stress. FXR-null mice generated enhanced oxidative stress, which may be attributable to a continuously high level of hepatic BAs. On the other hand, FXR activation appeared to repress CYP2E1 expression and attenuate oxidative stress, thus ameliorating liver injury in a murine model of alcoholic liver disease (ALD)[74,75]. FXR is proved to have anti-atherosclerotic effects as well[76]. Recently, down-regulation of hepatic FXR expression by endoplasmic reticulum (ER) stress has been proposed to be in close association with aging-induced fatty liver in mice, mainly through inhibition of hepatocyte nuclear factor 1 alpha (HNF1α)

transcriptional activity[53]. In general, these findings suggest extra mechanisms of FXR in treating NAFLD.

FXR AGONISTS IN TREATING NAFLD

Up to date, no efficient treatments are available for management of NAFLD. As FXR plays critical roles in mediating metabolic homeostasis and inhibiting inflammatory response, it is emerging as an ideal target for treatment of NAFLD. Numerous natural, semisynthetic, and synthetic FXR agonists have shown protective role in animal models and patients with NAFLD.

GW4064 is a non-steroidal synthetic FXR agonist. Activation of FXR by GW4064 suppressed weight gain and attenuated hepatic inflammation in C57BL/6 mice fed with either HFD or high-fat and high-cholesterol diet. GW4064 treatment also repressed diet-induced hepatic steatosis as evidenced by lower TG and FFA level in the liver, possibly due to markedly reduced lipid transporter CD36 expression. In this model, GW4064 improved hyperinsulinemia and hyperglycemia *via* decreasing PEPCK and G6pase[49]. Adiponectin and its receptors are two important factors in treatment of NAFLD. A recent study showed that treatment of GW4064 can up-regulate the expression of PPARγ2, adiponectin, adiponectin receptor 2 (adipoR2) in 3T3-L1 preadipocytes and adipoR2 in HepG2 cells, indicating that FXR agonist has a therapeutic potential in NAFLD[77]. GW4064 strongly induced FGF19 and inhibit CYP7A1, in which the hepatic FGF19/FGFR4/Erk1/2 pathway played a key role, which is independent of SHP. In addition to inducing FGF19 in the intestine, BAs in hepatocytes may activate the liver FGF19/FGFR4 signaling pathway to inhibit BA synthesis and prevent accumulation of toxic bile acid in human livers[35].

Obeticholic acid (OCA or INT-747, 6α-ethyl-chenodeoxycholic acid) is a semisynthetic derivative of the primary human bile acid chenodeoxycholic acid, and the natural agonist of FXR. Administration of OCA reversed hepatic steatosis and insulin resistance in Zucker (*fa*/*fa*) obese rats, protecting against body weight gain and fat deposition in liver and muscle, due to FXR-induced lipogenesis and gluconeogenesis decrease[19]. OCA can inhibit NF-κB-mediated hepatic inflammation, however, the anti-inflammatory effect of OCA are not liver-specific, OCA treatment can also reduce intestinal inflammation and permeability in experimental models of colitis[78]. Also, in primary rat HSCs, 6E-CDCA reduced thrombin-induced up-regulation of α1 (I) collagen, α-SMA and TIMP-1/2 mRNA expression and protected against fibrosis[79]. In a phase 2 clinical trial in patients with type 2 diabetes mellitus and NAFLD (ClinicalTrials.gov, No. NCT00501592), administration of 25 or 50 mg OCA for 6 wk was well tolerated. OCA was found to increase insulin sensitivity and significantly decrease levels of γ-glutamyltransferase and alanine aminotransferase (ALT). Markers of liver inflammation and fibrosis were also decreased in these patients[80].

WAY-362450, a synthetic potent FXR agonist, could attenuate hepatic inflammation and fibrosis in MCD diet induced NASH mice[20]. WAY-362450 treatment was also found to attenuate oxidative stress in a murine model of ALD[75]. Furthermore, treatment of obese *db*/*db* mice with INT-767, a dual FXR/TGR5 (a G-protein-coupled bile acid receptor) agonist, significantly improved the histologic features of NASH, resulted from recruitment of anti-inflammatory Ly6C^{low} monocytes to the liver, directly down-regulated the expression of Ly6C on bone-marrow derived monocytes and decreased production of pro-inflammatory cytokines by macrophages. In addition, INT-767 increased interleukin (IL)-10 production and enhanced hepatic expression of genes associated with alternatively activated macrophages. The data suggested INT-767 as a potential treatment target of NAFLD due to coordinating the immune phenotype of monocytes and macrophages[81]. In another study, INT-767 treatment markedly decreased cholesterol and TG levels in diabetic mice[82].

CONCLUSION

The data presented suggest that FXR plays crucial roles in mediating multiple target genes associated with bile acid, lipid and glucose metabolism and has beneficial effects on inflammation response, thus can partly interpret the pathogenesis of NAFLD. Accumulative data prove that targeting FXR may be beneficial in the prevention and treatment of NAFLD. However, some reports showed opposite results. For instance, the role of FXR in regulating HDL metabolism is still under debate, and need to be further evaluated. Some studies demonstrated different results, as FXR$^{-/-}$ mice had increased plasma HDL-cholesterol[44], and blockage of FXR activity also displayed reduced serum LDL levels and increased HDL levels[83], while activation of FXR by GW4064 suppressed apolipoprotein A-I transcription and reduced serum HDL levels[84]. On the other hand, FXR deficiency was shown to protect from excessive body weight gain in both genetic (*ob*/*ob*) and diet-induced obesity murine models and improve hyperglycemia and impaired glucose tolerance[85]. The same result also emerged in aging FXR deficient mice, and the reduced body weight gain is most likely explained by the increased energy expenditure[15]. In line with this, another study showed that activation of FXR with GW4064 was not useful for long term management of the metabolic syndrome, as it reduced the BA pool size and subsequently decreased energy expenditure, translating as weight gain and insulin resistance[86].

In summary, research on FXR has provided new opportunities to elucidate the pathogenesis of NAFLD and to develop effective treatment. Although activation of FXR by specific agonists could be an attractive pharmacological strategy for managing NAFLD, attention needs to be paid to several undesirable contradictory results, which remain to be elucidated. Since most evidence comes from preclinical studies, more clinical evidence is

urgently needed to establish treatments of FXR agonists for NAFLD.

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REVIEW

Pulmonary manifestations of inflammatory bowel disease

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Abstract

Extraintestinal manifestations of inflammatory bowel disease (IBD) are a systemic illness that may affect up to half of all patients. Among the extraintestinal manifestations of IBD, those involving the lungs are relatively rare and often overlooked. However, there is a wide array of such manifestations, spanning from airway disease to lung parenchymal disease, thromboembolic disease, pleural disease, enteric-pulmonary fistulas, pulmonary function test abnormalities, and adverse drug reactions. The spectrum of IBD manifestations in the chest is broad, and the manifestations may mimic other diseases. Although infrequent, physicians dealing with IBD must be aware of these conditions, which are sometimes life-threatening, to avoid further health impairment of the patients and to alleviate their symptoms by prompt recognition and treatment. Knowledge of these manifestations in conjunction with pertinent clinical data is essential for establishing the correct diagnosis and treatment. The treatment of IBD-related respiratory disorders depends on the specific pattern of involvement, and in most patients, steroids are required in the initial management. Corticosteroids, both systemic and aerosolized, are the mainstay therapeutic approach, while antibiotics must also be administered in the case of infectious and suppurative processes, whose sequelae sometimes require surgical intervention.

Key words: Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Lung diseases

Core tip: The clinicopathological patterns of pulmonary involvement in inflammatory bowel disease (IBD) consist of airway disease, lung parenchymal disease, thromboembolic disease, pleural diseases, enteric-pulmonary fistulas, and pulmonary function test abnormalities. The treatment of IBD-related respiratory disorders depends on the specific pattern of involvement, and in most patients, steroids are required in the initial management. This review focuses on the pulmonary manifestations of IBD in an attempt to avoid further health impairment and to alleviate symptoms by prompt recognition and treatment.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic inflammatory diseases of unknown etiology that commonly involve the gastrointestinal tract[1]. Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of chronic IBD. Extraintestinal and systemic manifestations occur commonly in patients with IBD (21%-41%)[2-4], increase with duration of intestinal disease, and affect most organ systems[5]. The extraintestinal manifestations are a significant cause of morbidity and may be particularly distressing for the patient[6]. Extraintestinal manifestations are more common in CD and may include cutane-

ous (pyoderma gangrenosum and erythema nodosum), ocular (anterior uveitis and episcleritis), hepatic (pericholangitis and fatty liver), and articular (peripheral and axial arthropathies) diseases[7]. Mouth ulcers and venous thrombosis also occur[8]. In contrast, pulmonary involvement is rare[9,10].

A possible link between UC and respiratory disease was described first by Turner-Warwick[11] in 1968, but it was not until the work of Kraft *et al*[12] in 1976 that respiratory involvement came to be included in the list of established complications of IBD. The authors described six adult patients with IBD who developed chronic bronchial suppuration with or without bronchiectasis. Following this report, many investigators described a similar pattern, and other manifestations of pulmonary involvement were described, including: interstitial pneumonitis, panbronchiolitis, bronchiolitis obliterans organizing pneumonia (BOOP), inflammatory tracheal stenosis, serositis, pulmonary vasculitis, apical fibrosis, Langerhan's cell histiocytosis, sarcoidosis, and conditions resembling Wegener's granulomatosis[13-23]. Respiratory diseases occurring in IBD may consist merely of a subclinical abnormal lung function or, in contrast, they may manifest as clear interstitial lung disease[15].

Pulmonary alterations are often overlooked, especially when respiratory symptoms are already present before the diagnosis of IBD. The true prevalence of lung involvement in IBD remains unknown and it seems rather variable, because in some series only a few cases of respiratory complications have been found[24]. However, it can be difficult to establish a relationship between respiratory diseases and IBD in patients who are already affected with pulmonary disease at diagnosis of IBD, or who are current smokers.

An ongoing bowel inflammation is not a prerequisite for the onset of respiratory alterations, because bronchopulmonary diseases that develop after colectomy have been reported[25]. Pulmonary abnormalities in IBD can present years after the onset of the bowel disease and can affect any part of the lungs. These may be overt or subclinical and do not correlate with the duration of IBD. The pulmonary manifestations are variously reported as occurring frequently during active disease, independent of disease activity, and even in post-colectomy patients[26,27].

The pathogenesis of IBD causing lung abnormalities involves some of the following mechanisms: both the colonic and respiratory epithelia share an embryonic origin from the primitive foregut and have columnar epithelia with goblet cells and submucosal mucus glands; the lungs and gastrointestinal tract contain submucosal lymphoid tissue and play crucial roles in host mucosal defense[28,29]. The similarity in the mucosal immune system causes the same pathogenic changes that may result from epithelial exposure to common antigens by inhalation and ingestion, leading to sensitization of the lymphoid tissue and inflammation[13]. The activated inflammatory cells in the bowel tissues are capable of producing several circulating cytokines such as interleukin (IL)-1, IL-2 and IL-6 and tumor necrosis factor (TNF)-α. These and other mediators can regulate the endothelial cell adhesion molecules, alter leukocyte migration, increase production of damaging reactive oxygen metabolites, and induce damage of lung parenchyma[6,30,31].

Although pulmonary involvement is well described in the literature, the evaluation and treatment of pulmonary disease associated with IBD remain a problem. The pulmonary associations of IBD are poorly characterized and early recognition is important[32]. Here, we review the pulmonary manifestations that are associated with IBD.

PULMONARY DISEASES ASSOCIATED WITH IBD

Airway diseases

Airway disease from the trachea to the bronchioles has been reported in association with IBD[29,33-50]. IBD was four times more prevalent among patients with airways disease compared with published local IBD prevalence in a retrospective analysis of outpatients over a 10-year period[51]. The pathogenesis of IBD-related airway disease is unknown, but it is clearly inflammatory in nature. Severe tracheal inflammation and obstruction are rare manifestations of IBD and correspond to the presence of irregularly friable and hemorrhagic tissue at endoscopy[52]. The tracheal epithelium is often ulcerated and is replaced by a thin layer of fibrin. The main symptoms are coughing, dyspnea, stridor and hoarseness[42]. Upper airway involvement comprises glottic/subglottic stenosis, tracheal inflammation and stenosis[13,40,42,43]. Most of this rare entity involves the trachea, presenting with shortness of breath, dysphonia, and cough[45,46,53]. It can often be identified by history, complemented by a clear X-ray film and obstructive pattern on pulmonary function testing. Laryngoscopic evaluation is necessary because airway compromise can occur. The mucosa may exhibit a cobblestone appearance similar to that seen in affected intestines[54]. Chest radiographs and computed tomography (CT) may show narrowing of any portion of the trachea, with circumferential tracheal wall thickening on CT[27]. No predilection seems to exist for specific gender or type of bowel disease.

Large airway disease, strongly associated with UC[18], is the most common presentation of pulmonary manifestations. Bronchial inflammation and suppuration are the most common manifestations of pulmonary involvement in IBD and include chronic bronchitis and bronchiectasis in which bronchial dilatation is visualized on chest X-ray or CT scan. Bronchiectasis is the most commonly reported entity and is noted in 66% of cases of IBD involving the large airways[13,34,35,39,50]. Infrequently, in IBD patients developing new, persistent and unexplained symptoms of respiratory disease, particularly chronic productive cough, the presence of bronchiectasis may be demonstrated[18]. The majority of patients with bronchiectasis have UC. IBD is inactive in many cases and curiously, in 60% of patients, the symptoms develop a few days to a few weeks following colectomy[39,42,55]. The second most common

large airway disease in IBD is chronic bronchitis, which is distinguished from bronchiectasis only by the degree and extent of pulmonary abnormality[56], and further abnormalities include suppurative large airway disease and acute bronchitis. The main symptom is chronic cough with purulent sputum poorly responsive to antibiotics[57]. Bronchial biopsy shows similar features: squamous cell metaplasia in the mucosa that is sometimes infiltrated by neutrophils and a dense cuff of lymphocytes and plasma cells infiltrating the submucosa[8].

Clinically, small airway disease is less frequently reported and is described as occurring in isolation from large airway disease. However, the recent advent of high-resolution CT has increased the detection of small airway involvement in these patients. These abnormalities seem to occur earlier in the course of the disease and at a younger age than large airway disease[41,47-49,58-60]. Moreover, small airway disease is more frequently apparent before the onset of IBD than other airway diseases[48]. CT shows bronchiolar wall thickening, mucoid impaction, centrilobular ground-glass nodules, and mosaic attenuation because of air trapping, and some patients have normal pulmonary function test (PFT) findings[32].

Bronchiolitis is an inflammatory and potentially fibrosing condition affecting mainly the intralobular conducting and transitional small airways[61]. Among small airway diseases are associated with IBD, bronchiolitis is the most frequently detected[62,63]. Chronic bronchiolitis contributes to morbidity and/or mortality if it persists and/or progresses to diffuse airway narrowing and distortion or complete obliteration. Bronchiolitis in specific settings leads to bronchiolectasis, resulting in bronchiectasis. The main symptoms of bronchiolitis associated with IBD include mild productive cough and chronic bronchorrhea; wheezes are heard at auscultation. Small airway involvement can precipitate abnormalities on PFTs. Histological samples show varied patterns ranging from nonspecific fibrosing and stenosing bronchiolitis to an inflammatory lesion indistinguishable from the original description of panbronchiolitis[8]. The CT appearances coupled with the evaluation of pulmonary function parameters usually lead to the diagnosis.

In IBD-related large airways disease, steroid drugs are effective, but recommendations for their use including dosage, duration and route of administration remain empirical. Steroids are the major therapy although some patients do not require systemic therapy. Clinical improvement with inhaled steroids alone or in combination with systemic steroids has been reported[8,29,43,64]. Ineffectiveness of inhaled corticosteroids may be due to airways filled with inspissated secretions, in which case either topical corticosteroids via bronchoalveolar lavage (BAL) or systemic corticosteroids are recommended[57]. Broadly speaking, inhaled steroids seem more effective and are better tolerated than oral steroids. Rarely, other forms of immunomodulation have been used to treat IBD-related airway disease[65]. Small airway disease is usually refractory to inhaled steroids, and the improvement brought about by oral steroids ranges from slight to modest[8]. Lung transplantation has been required in some cases. Surgery of the colon, which may aggravate prior airway disease[37], is not recommended for treatment of airway disease.

Lung parenchymal diseases

Lung parenchymal disease associated with IBD is relatively uncommon. Analysis of diffuse lung disease in IBD patients is further confounded by documented pulmonary sequelae to various medical therapies used to treat IBD. In contrast to other extraintestinal manifestations, lung parenchymal disease associated with IBD is seen more commonly with UC than CD[8]. Age of onset varies, and there is a slight female predominance.

BOOP is the most commonly reported parenchymal manifestation of IBD[63,66-69]. BOOP is often caused by inhalation injury, or results from a post-infection origin or drugs and may present acutely or subacutely with fever, cough, dyspnea and pleuritic chest pain[15,70,71]. Chest radiography shows focal to diffuse peripheral predominant airspace opacities. CT shows scattered, nonsegmental, unilateral, or bilateral foci of consolidation, ill-defined centrilobular nodules, and large irregular nodules. It can be associated with other autoimmune diseases such as rheumatoid arthritis, lupus and Wegener's granulomatosis. Dyspnea and cough are the most common presenting symptoms. Systemic steroids are recommended for treatment but BOOP may also remit without treatment in a minority of cases[13].

Other forms of parenchymal disease that may be related to IBD or drug toxicity are eosinophilic pneumonia and nonspecific interstitial pneumonitis. Although interstitial disease most commonly involves drug-induced reactions with mesalamine and sulfasalazine, a small number of unrelated cases of fibrosing alveolitis and eosinophilic pneumonia have been reported[72-77]. On CT, peripheral consolidation predominates in cases of eosinophilic pneumonia, whereas nonspecific interstitial pneumonitis shows ground-glass opacities, interlobular septal thickening, and irregular linear opacities[78]. The interstitial lung infiltrates have been proven histologically to be either pulmonary vasculitis[79-81] or more often granulomatous disease[82-85].

Pulmonary nodules have been infrequently reported in patients with IBD. Histologically, these lesions have been reported to be necrobiotic, granulomatous, or otherwise[86]. Necrobiotic nodules, composed of sterile aggregates of neutrophils with necrosis, may also be seen in rheumatoid arthritis, Wegener's granulomatosis, or septic pulmonary emboli, and should be differentiated from malignancy and infection. An infectious origin should be excluded because necrobiotic nodules will respond to steroids but not to antibiotics. Sarcoidosis and CD are both granulomatous diseases, of the lung and bowel, respectively. It is not surprising that these two diseases may simultaneously appear in the same patient, with pulmonary involvement[87], even though this happens rarely and the two diseases usually follow an independent clinical course[88]. An infectious cause, specifically atypical *Mycobacterium* has been postulated to contribute to granuloma

formation in both sarcoidosis and CD, and has even been detected in tissues from patients with both diseases[89].

The manifestations of lung parenchymal disease in IBD usually respond dramatically to inhaled and/or systemic steroids. Steroids administered orally lead to marked improvement in patients with interstitial lung disease, BOOP, pulmonary infiltrates with eosinophilia, and necrotic nodules. Intravenous steroids are required in the initial management of life-threatening complications such as extensive interstitial lung disease. The addition of cyclophosphamide or infliximab may show rapid clinical and radiological response and are well tolerated in some cases[90,91].

Thromboembolic diseases

IBD is a chronic inflammatory condition, characterized by microvascular and macrovascular involvement. Inflammation and immune response could lead to endothelial dysfunction, which is the earliest stage of the atherosclerotic process[92]. Chronically inflamed intestinal microvessels of IBD patients have demonstrated significant alterations in their physiology and function compared with vessels from healthy and uninvolved IBD intestine[93]. Thromboembolism is an extraintestinal manifestation and an important cause of mortality in IBD[94]. The incidence of thromboembolic events in IBD patients is three to four times higher than in age-matched control subjects[95,96]. It happens at an earlier age than in non-IBD patients. The majority of thromboembolic events among IBD patients are venous thromboembolism, manifested as either deep venous thrombosis or pulmonary embolism, but arterial thromboembolism and venous thrombosis at unusual sites have also been reported[97]. Prothrombotic risk factors in IBD patients could be distinguished as acquired, such as active inflammation, immobility, surgery, steroid therapy, and use of central venous catheters, and inherited[93].

The risk of thromboembolism appears to be multifactorial and related to mucosal inflammatory activity in most patients. Pulmonary embolism should be always considered in IBD patients with breathing difficulties. However, the diagnosis of venous and arterial thromboembolism is extremely challenging and requires a high degree of vigilance. Deep vein thrombosis and pulmonary embolism may be clinically silent or manifest with only a few specific symptoms. Up to one-third of thromboembolic events in this population occur while IBD is quiescent, suggesting an unknown risk factor that is unrelated to treatment or disease activity[13]. The pathogenesis of increased thrombotic risk among patients with IBD is unclear. About 80% of IBD patients have active disease when pulmonary embolism occurs[98]. Early diagnosis plays a central role in optimizing the therapeutic intervention and reducing the risk of short-term and long-term thrombosis-associated complications. The decision regarding the duration of systemic anticoagulation must take into account the individual risk of intestinal bleeding[99].

Pleural diseases

Rarely, IBD involves the pleural space and pericardium, causing inflammatory exudative pleural and/or pericardial effusions[100,101]. This is a relatively rare presentation of the uncommon and probably under-reported and under-recognized pulmonary extraintestinal manifestations of IBD[102]. Pleuropericardial inflammatory disease and effusion can be directly related to IBD, its complications, associated infections, or the medications used to treat it[103]. Most patients are young, male, and have UC during the quiescent phase of the disease. The manifestations of pleural disease can be classified as: pneumothorax[104], pleural thickening[105], pleuritis, and pleural effusion[106]. Pleural fluid directly related to IBD is usually unilateral, an exudate with neutrophils, and may be hemorrhagic. Mesalazine may also induce lupus-like symptoms, such as arthralgia, pericarditis, tamponade, and/or pleural effusion, with positive antinuclear antibody[107]. It is important to evaluate pleural effusion and rule out other etiologies before making this diagnosis. Pleural or pericardial biopsies are rarely necessary, and probably show nonspecific acute and chronic inflammatory changes[103]. Although the specific pathophysiology of pleuropericardial disease in patients with IBD remains unclear, the response to systemic steroids is usually adequate. However, pleural drainage may be required occasionally.

Enteric-pulmonary fistulas

Fistula formation is frequent in CD and occurs in 33% of patients[107]. Most of the fistulas appear in the perineal area[108]; to date, only a few reports (mostly as single cases) are available on the occurrence of enteric-pulmonary fistulas in IBD, such as colobronchial[109-112], ileobronchial[113], and esophagobronchial[114,115] fistulas. In most cases, colobronchial fistulas extend from the splenic flexure in the colon to the lower lobe of the left lung. This is likely due to the anatomical proximity between the two structures. However, Mercadal *et al*[116] reported a rare case of right-sided colobronchial fistula in a 47-year-old, severely malnourished man with a history of regional enteritis and recurrent right lower and middle lobe pneumonia, medically managed with the addition of the immunomodulator infliximab prior to surgery.

Diagnosis of fecopneumothorax is based on meticulous clinical examination and additional diagnostic procedures. Recurrent pneumonia with feculent sputum in patients with CD should raise suspicion of colobronchial fistula. Once the abnormal connections between the bowel and respiratory tract are suspected, an enema using water-soluble contrast medium certainly helps to confirm the presence of fistulas[110]. Abdominal and thoracic CT scan or magnetic resonance imaging could provide additional information about the stage of the disease and exclude the presence of abscess or fluid collection in the abdominal cavity. Colopleural fistula and fecopneumothorax are rare but life-threatening complications of CD[117]. Surgical treatment is mandatory as soon as the diagnosis is established[118].

Pulmonary function test abnormalities

PFT abnormalities are found frequently in patients with

IBD without presence of any respiratory symptoms and lung radiograph findings[20]. IBD patients show significantly decreased lung function tests in comparison to healthy controls. In a review including over 600 patients with UC, more than 50% of patients showed abnormal PFT results when compared to healthy controls, and the decrease in diffusion capacity of the lung for carbon monoxide (DLCO) was the most common defect[15]. Various studies testing pulmonary function in patients with IBD have revealed a spectrum of abnormalities including restrictive disease, obstructive disease, bronchial hyperresponsiveness and hyperinflation as well as a decreased diffusion capacity of the lung[119-133]. The severity and frequency of these PFT abnormalities that are detected even in the remission periods increase with disease activity. There is no difference between UC and CD in PFTs[51], and smoking status is not predictive of these abnormalities.

The most commonly described abnormality is a decrease in lung diffusion capacity[66]. In most studies, this alteration could not be predicted by current or past smoking status, occupational history, or current medication use. Lung transfer factor for carbon monoxide abnormalities is related to the degree of disease activity[122]. Pulmonary involvement in IBD is often asymptomatic and detectable only at the time of lung function investigation. This is further supported by the finding of Wallaert *et al*[134] of a high proportion of latent lymphocytic pulmonary alveolitis in the BAL of 18 consecutive patients with CD; all free from respiratory symptoms and showing normal chest X-ray. Therefore, PFT may be used as a noninvasive diagnostic procedure in determining the activation of IBD and might aid early diagnosis of latent respiratory involvement[135]. Early recognition is important, because PFT abnormalities can be steroid responsive[136].

PFT studies in IBD suggest that subclinical pulmonary disease may be present in a large subpopulation of patients. A high degree of suspicion is necessary to detect the pulmonary abnormalities in IBD, because a large proportion of symptom-free patients have abnormal findings on pulmonary function testing. Although the mechanism of these abnormalities remains unclear, it may be a result of the increased capacity of alveolar macrophages to produce superoxide anions, which has been shown in some patients with CD[137].

DRUG-RELATED LUNG DISEASES

Although drug-related diseases are not "proper" IBD-associated diseases, because IBD patients use several drugs for prolonged periods of time, it is not surprising that some of these may also cause problems to the lungs. Therefore, this type of pathology must be kept in mind for patients taking azathioprine (AZA), 6-mercaptopurine (6-MP), sulfasalazine, mesalamine, methotrexate, and anti-TNF-α.

AZA and 6-MP

AZA and 6-MP are therapeutic options for patients with moderate to severe IBD[138]. However, between 10% and 29% of patients treated with these drugs are forced to stop therapy due to side effects. Pulmonary toxicity due to these drugs has been reported infrequently in the literature, although interstitial pneumonitis[139], BOOP[140], chronic pneumonitis/fibrosis and pulmonary edema[141] have been described after use of AZA and 6-MP. Although rare, AZA and 6-MP can cause direct, dose-dependent and serious pulmonary toxicity[140,142]. The largest number of cases of lung toxicity related to AZA was described in seven patients undergoing renal allograft transplant immunosuppression with AZA[142]. Lung biopsies revealed interstitial pneumonitis in 5 patients and diffuse alveolar damage in 2; 3 patients died and the other 4 improved after stopping AZA, and in 2 of these patients, cyclophosphamide therapy was needed to resolve this side effect completely. Thus, it is important for clinicians to have a high index of suspicion for this adverse reaction, which occurs within 1 mo after purine analog use in IBD.

Sulfasalazine and mesalamine

Sulfasalazine and mesalamine are commonly used medications for the long-term treatment of IBD, and their side effects may be dose-related or idiosyncratic and should be differentiated from the respiratory involvement occurring in IBD and due to the underlying disease, although this is challenging because they share similar pathological features[47]. Commonly reported lung pathology related to the use of these compounds is mostly due to interstitial disease[54,128,143-145], although eosinophilic pleuritis[146], eosinophilic pneumonia[71,147-150], and bronchiolitis obliterans[67] have also been described. Patients present with progressive respiratory symptoms such as dyspnea, chest pain and cough, and radiographic abnormalities. Alternatively, sulfasalazine and mesalamine may induce asymptomatic lung injury more commonly than is presently suspected[151]. Although sulfasalazine or mesalazine-induced lung injury is a rare entity, its possibility should be fully considered in patients developing unexplained respiratory symptoms while on sulfasalazine or mesalazine therapy[150]. In most cases, symptoms appear after 2-6 mo of drug use, whereas in a few cases they appear after a few days or after many years. These pulmonary toxicities appear reversible after withdrawal of the drug, and in some cases, with the use of systemic corticosteroids[152,153].

Methotrexate

Methotrexate (MTX) may be useful in the treatment of IBD[154], but can cause adverse effects in the lungs, which in some cases are lethal[155]. The mechanism of MTX-induced lung pathology remains unclear. A hypersensitivity reaction was suggested by lung biopsy findings: interstitial pneumonitis, granuloma formation and bronchiolitis[156], and by BAL findings: lymphocytic alveolitis, increased eosinophils and reversed CD4/CD8 ratio[157], together with the clinical findings of fever, peripheral eosinophilia and response to corticosteroids. MTX may also cause pneumonitis[158], and abnormal ventilation is an early sign and should lead to further investigation[159]. The diagnosis of

MTX-induced lung disease is difficult because there are no pathognomonic findings and this condition may mimic other pulmonary diseases. The most frequent complaints include dyspnea, fever and nonproductive cough. PFTs show a restrictive picture with low CO diffusion capacity. MTX-related lung toxicity is potentially fatal, thus, regular monitoring of the status of the respiratory system in MTX-treated patients is necessary and patients should be instructed to report any new pulmonary symptoms without delay[160]. Besides supportive therapy, withdrawal of MTX seems to be a logical approach.

Biological therapy

Biological therapy with anti-TNF drugs such as infliximab, adalimumab and certolizumab has represented a significant advance in the treatment of IBD over the past few years[161-163]. However, serious side effects do occur, necessitating careful monitoring of therapy[164]. Several associated opportunistic infections have been observed as a result of suppression of T-cell-mediated immunity; the most frequent being tuberculosis[165-167]. Physicians should be aware of the increased risk of reactivation of tuberculosis in patients treated with anti-TNF agents and regularly look for usual and unusual symptoms of tuberculosis. Moreover, the use of biological therapy has been associated with *Pneumocystis carinii* pneumonia[168], as well as with other pulmonary infections (coccidiomycosis, histoplasmosis, aspergillosis, nocardia asteroids, actinomycosis and listeriosis)[169-173], especially in older patients[174].

Although infective complications are the most feared after the use of biological agents, these may induce other uncommon effects in the lung, such as acute respiratory distress syndrome[175], diffuse alveolar hemorrhage[176], non-bronchiolitis inflammatory nodular pattern of the lung[177], and interstitial lung disease[178-180]. Close observation of patients undergoing treatment with TNF inhibitors for evolving signs and symptoms of autoimmunity is required. Organ involvement is unpredictable, which makes correct diagnosis and management extremely challenging.

CONCLUSION

Pulmonary manifestations of IBD are being increasingly recognized. The involvement of the respiratory system in IBD, which can range from a simple defect of pulmonary function without symptoms, to fibrosing alveolitis with a greater risk of mortality, is relatively rare but sometimes potentially harmful. Early identification of latent pulmonary involvement is important to prevent future and more severe respiratory impairment and can be life-saving. The manifestations in the lung vary and often represent a confounding diagnostic problem. It is imperative for clinicians to maintain a high index of suspicion for the development of pulmonary disease in the setting of IBD in order to institute appropriate treatment early and avoid further complications and morbidity in IBD patients, and to recognize prompt treatment for these events. Steroids are effective in the majority of cases.

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MINIREVIEWS

Review of experimental attempts of islet allotransplantation in rodents: Parameters involved and viability of the procedure

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Abstract

The purpose of the present study was to organize the parameters involved in experimental allotransplantation in rodents to elaborate the most suitable model to supply the scarcity of islet donors. We used the PubMed database to systematically search for published articles containing the keywords "rodent islet transplantation" to review. We included studies that involved allotransplantation experiments with rodents' islets, and we reviewed the reference lists from the eligible publications that were retrieved. We excluded articles related to isotransplantation, autotransplantation and xenotransplantation, *i.e.*, transplantation in other species. A total of 25 studies related to allotransplantation were selected for systematic review based on their relevance and updated data. Allotransplantation in rodents is promising and continues to develop. Survival rates of allografts have increased with the discovery of new immunosuppressive drugs and the use of different graft sites. These successes suggest that islet transplantation is a promising method to overcome the scarcity of islet donors and advance the treatment options for type 1 diabetes.

Key words: Islet transplantation; Allograft; Immunosuppression; Type 1 diabetes; Islet grafts; Diabetes mellitus; Islet; Hyperglycemia

Core tip: This is an important systematic review for readers to analyze the different existing methodologies of islet allotransplantation. This article reviews all aspects of donors and recipients, the types and dosages of immunosuppressive therapy, graft survival time and evolution of the recipient's blood glucose. Therefore, the present article permits reproduction and improvement of the experiments involving islet allotransplantation in rodents to develop alternative therapies for type 1 diabetes.

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INTRODUCTION

It is estimated that 4% of the world population is affected by diabetes mellitus, of which 10% have type 1 diabetes[1]. Furthermore, the incidence of diabetes cases in Europe has increased, especially in children and teenagers, in whom the incidence of type 1 diabetes increased by 4% last year. One trend is the occurrence of type 1 diabetes mellitus at younger ages, between 10 and 14 years. Today, the disease is already at 0-5 years[2]. According to the IBGE-CENSUS-2010, there are currently 12054827

diabetics patients in Brazil[3]. Thus, approximately 1.2 million diabetics in Brazil may benefit from research aimed at improving treatment of type 1 diabetes.

Currently, insulin is the primary treatment method for diabetes. However, approximately 5%-10% of patients have severe and unexpected fluctuations in their blood glucose levels, resulting in multiple episodes of hypoglycemia, which has serious clinical consequences. In such cases, pancreas transplantation is an alternative treatment option that is already in clinical use. Another alternative option is islet transplantation, which is a less invasive therapeutic method but is still in development[3]. Regarding the effectiveness of treatment, some results showed 80% insulin independence within the first year in postoperative patients treated with islet transplantation[4]; however, the survival rate of islets remains low.

The scarcity of islets is a significant obstacle hindering the widespread use of islet allograft therapy. According to the Network of Organ Procurement and Transplantation, in 2011, only 1562 pancreases were recovered from 8000 donor organs available in the United States. Furthermore, many donated pancreases are not suitable for islet extraction or do not fit the selection criteria. It is also common for islets to be handled incorrectly. For these reasons, only a small number of islet transplantations can be carried out[5].

Some restrictions were found in the technical development of islet transplantation: the number of donor pancreases available for islet transplantation, below that required for healing the millions of people with type 1 diabetes; technical difficulties and the cost of islet isolation; poor durability of insulin independence; and autoimmunity and rejection after transplantation, which must still be overcome. It is therefore essential to develop an unlimited source of cells capable of secreting insulin in response to glucose and that can be transplanted with little or no need for systemic immunosuppression[6,7].

The purpose of the present study is to review experimental allotransplantation procedures that have been attempted in rodents to analyze the parameters involved and the viability of the procedure.

SEARCH PROCESS

Search process

The study was performed using the PubMed database to search for published articles containing the keywords "rodent islet transplantation". However, to filter the results, we searched PubMed records for the period January 2000-December 2013 using the following search terms for islet allotransplantation in rodents: "{rodent islet transplantation AND ["2000"(Date-Completion): "3000"(Date-Completion) AND (allotransplantation) NOT porcine] NOT tilapia} NOT nonhuman primate".

This ensured that articles discussing transplantation in porcine, tilapia and nonhuman primates (more common species used for transplantation) were excluded from the review to focus on those articles related to allotransplantation in rodents. Following the PubMed search, we reviewed the references from the publications retrieved and obtained the entire text of publications that could potentially be included in the systematic review. Unpublished studies and letters were ignored. Studies that did not have a full text available in English were purchased for review.

Eligible studies were selected for analysis based on the following inclusion criteria: (1) studies must be related to allotransplantation; (2) the species studied must be rodent species; and (3) articles must be relevant and the information up to date.

The review was written in English, and the relevant information, such as donor/recipient, immunosuppression, allotransplantation site, graft survival time, glucose variations and diabetes induction method, was organized into tables.

DATA ABSTRACTION

The authors abstracted the characteristics of the study, such as the source(s) for experimental (*e.g.,* medical records and clinical databases) and relevant data, into the tables-donor/recipient; immunosuppression; allotransplantation site; graft survival time; glucose variations; and diabetes induction method.

SEARCH RESULTS

A total of 2650 articles from 2000 to 2013 were found. Only 25 articles were related to allotransplantation. These articles were selected based on their relevance and updated information (Tables 1-6; Figure 1A and B).

DISCUSSION

Islet transplantation has the potential to provide an adequate supply of insulin to the transplanted patient and provide a solution to the problem of islet donor shortage[6].

The first successful islet transplantation for the surgical treatment of diabetes occurred in 1990 by Shapiro *et al*[7]. Insulin independence was achieved in a patient with type 1 diabetes at one month post-transplant. However, many technical difficulties were found that needed to be overcome to continue the development of this technique and reproduce this experiment. In the decade between 1991 and 2000, 450 islet transplantation attempts were made in type 1 diabetic patients, with a success rate of only 8%. Fifty percent of successful cases were reported when patients had become diabetic because they were undergoing a pancreatectomy.

Then, in 1999/2000, Shapiro *et al*[7] successfully achieved insulin independence in 7 diabetic patients by performing experiments based on the modified Edmonton protocol[2,7].

Islet transplantation has increasingly been shown to reduce morbidity 20-fold compared to pancreas transplant because it is surgically less invasive[8].

In the present study, we reviewed studies consisting of rodents similar in age and weight undergoing allograft transplantation. Strains of mice aged 6-12 wk and weighing 200 g to 350 g were used. According to these studies

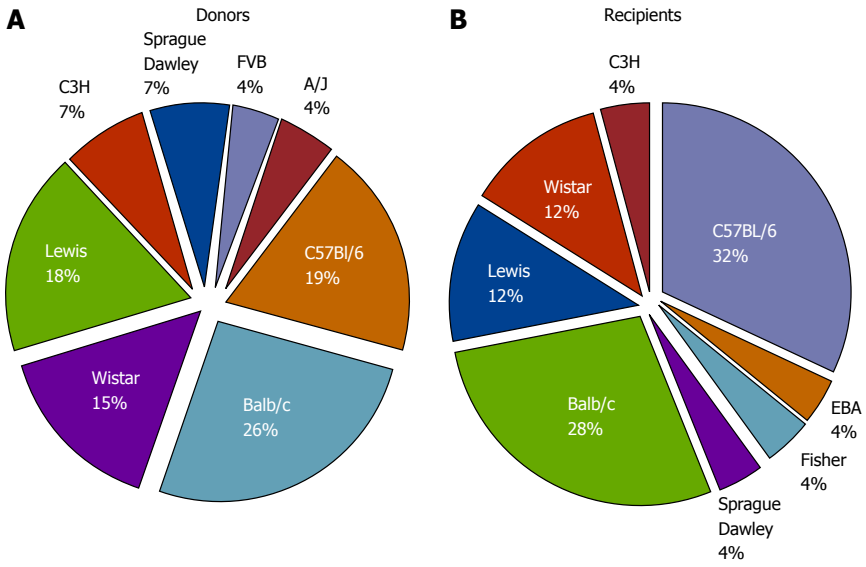

Figure 1 Quantitative and comparative analysis of the different donor strains (A) and recipient strains (B).

Table 1 Description of the experimental studies on allografts in rodents

Ref.	Donor/recipient	Immunosuppression	Allotransplantation site	Graft survival time
Fotiadis *et al*[9]	Lewis→ Wistar	Mycophenolate mofetil (MMF) and Cyclosporine A (CsA)	Spleen	N/A
Merani *et al*[10]	Lewis →Wistar	AEB-071 (Protein kinase C inhibitor) + CsA, CTLA4-Ig, MMF	Kidney capsule	100 d
Nishimura *et al*[11]	C57BL/6 → Balb/c	Tacrolimus	Nonmetallic dorsal skinfold chamber	N/A
Makhlouf *et al*[12]	C57BL/6/Balb/c	Blockade of CD28:B7 and anti-CD40L; CTLA-4	Kidney capsule	1 wk
Salazar-Bañuelos *et al*[13]	Wistar →Sprague Dawley	No immunosuppression	Medullary channel	21 d
Wee *et al*[14]	Lewis → Fisher	CsA + Tautomycetin (synergist)	Liver (portal vein)	Control group - 5.2 ± 0.5 d TMC - 5.1 ± 0.9 d TMC (0.03 mg/kg) + CsA (5 mg/kg) - > 41 d TMC (0.1 mg/kg) + CsA (5 mg/kg) - 103.8 ± 56.8 d
Plesner *et al*[15]	Balb/c →EBA	No immunosuppression	Kidney capsule	60 d
Watanabe et al[16]	Balb/c → C57BL/6	Tacrolimus and DHMEQ (NF-κB inhibitor)	Kidney capsule	100 d
Gysemans *et al*[17]	Balb/c→ C57BL/6	No immunosuppression	Kidney capsule	9.2 ± 4.9 d (Autoimmune diabetes) 15 ± 3 d (Not chemically diabetic autoimmune)
Xekouki *et al*[18]	Wistar→Lewis	CsA and MMF	Spleen (parenchyma)	8 d (CsA) 10.92 d (MMF 1) 11 d (MMF 2)
Baker *et al*[19]	A/J→ C57Bl/6J	Monoclonal antibody antiBIP-10	Kidney capsule	19.7 ± 2.3 d (C57Bl/6J) 20.2 ± 2.7 d (CXCR3-/-C57Bl/6J
Li *et al*[20]	FVB→ Balb/c	No immunosuppression	Kidney capsule	N/A
Vieiro *et al*[21]	C57Bl/6→ C3H	Tritiated thymidine (preoperative) and CsA	Subcutaneous	N/A
Neuzillet *et al*[22]	C3H → Balb/c	No immunosuppression	Kidney capsule	13.8-27.5 d
Melzi *et al*[23]	C57Bl/6 → Balb/c	Rapamycin+ FK506+ anti-IL-2Ra chain mAbs and rapamycin+IL-10	Kidney capsule	> 100 d
Fiorina *et al*[24] Fan *et al*[25]	Balb/c→ C57Bl/6 C57Bl/6 → Balb/c	No immunosuppression LTß R-Ig, CTLA4-Ig or LTR mAb anti mouse	Kidney capsule	14 d LTβ R-Ig- 27 d CTLA4-Ig- 55 d LTβ R-Ig+CTLA4-Ig - > 100 d LTR mAb anti mouse - 11 d

Jung *et al*[26]	Balb/c → C57Bl/6	CD154 mAb (MR1) anti mouse and ROS-A (Reactive Oxygen Specie - A)	Kidney capsule	ROS-A - 53 d MR1 - 82 d ROS-A+MR1 - > 160 d
Påhlman *et al*[27]	Balb/c → C57Bl/6J	AR-C117977 (10 or 30 mg/kg) or CsA 20 mg/kg	Kidney capsule	CsA - 16 d AR-C117977, 10 mg/kg - >100 d AR-C117977, 30 mg/kg -29,33 d
Wang *et al*[28]	Balb/c → C57Bl/6	B7-H4 and Ad-LacZ	Kidney capsule	B7-H4 - approximately 60 d Ad-LacZ - approximately > 20 d
Chen *et al*[29]	Sprague Dawley → Lewis	No immunosuppression	Intra-abdominal	8 wk
Giraud *et al*[30]	C3H → Balb/c	No immunosuppression	Kidney capsule	SCOT + PEG 20 kDa 310 g/L - 20 d, CMRL-1066 + 1% BSA - 17.5 ± 1 d, Solution UW - 17.2 ± 0.4 d, SCOT without PEG - 14 ± 0.9 d Solution HBSS + 0.5% BSA - 14 ± 0.7 d
Qi *et al*[31]	Wistar/Lewis → Lewis	No immunosuppression	Intraperitoneal (Macroencapsulated) Kidney capsule (not macroencapsulated)	24 wk (Macroencapsulated) 48 h (not macroencapsulated)
Potiron *et al*[32]	Wistar → C57Bl/6	CTLA4 Ig or CD40 Ig	Kidney capsule	24.3 ± 9.7 d
Jahr *et al*[33]	Lewis → Wistar	Anti-rat antilymphocyte serum	Liver (portal vein)	

N/A: Not available.

Table 2 Sites of islet infusion based on the literature from the PubMed database

Sites of islet infusion	Literature from the PubMed database
Kidney capsule	70%
Liver	23%
Other sites[1]	7%

[1]Subcutaneous, bone marrow, sub-retinal space, sub-conjunctival space, spleen, intraperitoneal cavity and sub-mucosal space of the duodenum.

(Table 3), C57BL/6 (B6) and Balb/c were the most commonly used strains in allograft experiments as both islet donors (23.8% and 16.67%) and as islet recipients (28.57% and 16.67%). However, no significant difference was observed in the results obtained using other strains.

The efficacy of combined immunosuppressive drug therapy on islet transplantation in rodents has been widely studied. Among these studies, Fotiadis *et al*[9] tested the effects of cyclosporine A (CsA) given in combination with mycophenolate mofetil (MMF) and found that the survival rate increased significantly compared to the isolated use of CsA and MMF; this observation was presumably due to its lower toxicity in the combined regimen.

Nishimura *et al*[11] conducted studies with tacrolimus and demonstrated a suppression in vascular endothelial growth factors, protein kinase 14 activated by mythogen, tissue factor F, specific cyclin D1 for G1/S cell division and protein kinase 4. Thus, they concluded that the drug inhibits pancreatic islet revascularization. However, hypoxia-inducible factor 1 alpha (HIF1A) was not observed. Thus, there was a minor engraftment of islets and subsequent degeneration. Furthermore, no differences were observed in gene expression between the control group and the group receiving tacrolimus.

Wee *et al*[14] studied the effects of tautomycetin and concluded that it does not affect the viability of the islets and spleen, but it is capable of inhibiting the proliferation of T cells. When tautomycetin was combined with subtherapeutic doses of CsA, it led to increased survival of islets. A dose of CsA of 15 mg/kg prolonged the survival of islets the longest. Thus, the mixture of tautomycetin with CsA or other calcineurin inhibitors increased islet survival.

Merani *et al*[10] demonstrated that inhibition of PKC using the new drug AEB -071 slowed the rejection of islet allografts in rodents. Furthermore, addition of CsA therapy with 5 mg/kg AEB prevented graft rejection in 80% of the rats transplanted by immunosuppressive action of the complement system and had no toxic effects.

Watanabe *et al*[16] conducted studies with DHMEQ, an inhibitor of NF-κB, and concluded that the proinflammatory responses activated by HMGB1 were reduced. Moreover, the immunosuppression allows allograft acceptance even in cases where only a few islets were transplanted.

Xekouki *et al*[18] analyzed the effects of CsA and MMF. Their results suggest a beneficial effect of MMF in maintaining the architecture of the islets without prominent side effects in other organs, such as the kidneys or liver.

Baker *et al*[19] studied *CXCR3* gene deletion and αIP-10 antibody therapy and concluded that they modulate post-transplant lymphocytic infiltration into the graft and contribute to prolonging allograft survival.

Fan *et al*[25] concluded that the simultaneous blockade of LIGHT and CD28 prolongs graft survival because of a synergistic effect; the presence of T-regulatory cell activity develops donor-specific immunological tolerance. Moreover, prevention of allograft rejection and induction of donor-specific tolerance in lymphocyte-sufficient recipients can be achieved by local cotransplantation of the

Table 3 Comparative analysis of the different types of rodents used and their basic characteristics-Age and Weight

Ref.	Donor/recipient	Age	Weight
Fotiadis *et al*[9]	Lewis→ Wistar	N/A	220 g-300 g
Merani *et al*[10]	Lewis→ Wistar	N/A	200 g (male)/150 g (female)
Nishimura *et al*[11]	C57BL/6 → Balb/c	9-12 wk /8-12 wk	N/A
Makhlouf *et al*[12]	C57BL/6→ Balb/c	6-8 wk (male)/ N/A	N/A
Salazar-Bañuelos *et al*[13]	Wistar→ Sprague Dawley	N/A	260-326 g
Wee *et al*[14]	Lewis→ Fisher	10-12 wk	N/A
Plesner *et al*[15]	Balb/c→ EBA	8-10 wk /N/A	N/A
Watanabe *et al*[16]	Balb/c→ C57BL/6	10-14 wk /male	N/A
Gysemans *et al*[17]	Balb/c→ C57BL/6	(8-21 d) → (> 180 d)	N/A
Xekouki *et al*[18]	Wistar→Lewis	N/A/male	220-300 g
Baker *et al*[19]	A/J→ C57Bl/6J	8-12 d/male	N/A
Li *et al*[20]	FVB→ Balb/c	8-12 wk	N/A
Vieiro *et al*[21]	C57Bl/6→ C3H	N/A	N/A
Neuzillet *et al*[22]	C3H → Balb/c	N/A	N/A
Melzi *et al*[23]	C57Bl/6 → Balb/c	9 wk (female) → 9 wk (female)	20-22 g
Fiorina *et al*[24]	Balb/c→ C57Bl/6	N/A	N/A
Fan *et al*[25]	C57Bl/6 → Balb/c	N/A - adults (female)	N/A
Jung *et al*[26]	Balb/c → C57Bl/6	12 wk (male) → 12 wk (male)	25-30 g
Påhlman *et al*[27]	Balb/c → C57Bl/6J	N/A - (female)	N/A
Wang *et al*[28]	Balb/c → C57Bl/6	8-10 wk (female) → 8-10 wk	N/A
Chen *et al*[29]	Sprague Dawley → Lewis	N/A (male)	250-350 g → 196 ± 15 g
Giraud *et al*[30]	C3H → Balb/c	6 wk	N/A
Qi *et al*[31]	Wistar/Lewis → Lewis	9-10 wk (male)	250-300 g
Potiron *et al*[32]	Wistar → C57Bl/6	N/A (male)	200-300 g
Jahr *et al*[33]	Lewis → Wistar	N/A (male) → N/A (male)	310-330 g→ 215-245 g

N/A: Not available.

Table 4 Analysis of the immunosuppressant drugs used at international islet transplantation research centers

Immunosuppressant	Number of centers using the immunosuppressant (based on data from the literature)
CsA	6
MMF	3
CTLA4 Ig	4
CD40 Ig	2
NF-kB Inhibitor (DHMEQ)	1
Anti-CD154 mAb (MR1)	2
Tritiated thymidine	1
Tacrolimus	1
Blockade of CD28:B7	1
Tautomycetin	1
Protein Kinase C Inhibitor (AEB-071)	1
Monoclonal antibody anti-BIP-10	1
Rapamycin+FK506+anti-IL-2Ra chain mAbs, n31 and rapamycin+IL-10; n29	1
LTβ R-Ig	1
LTR mAb	1
ROS-A	1
AR-C117977	1
B7-H4 and Ad-LacZ	1
Anti-rat antilymphocyte serum	1
No immunosuppression	9

allografts with regulatory T cells.

Jung *et al*[26] concluded that the combination of Ros A and MR1 in a murine allogeneic islet transplantation model prolonged graft survival compared to the MR1-alone treatment group.

Påhlman *et al*[27] evaluated the immunosuppressive limitations of AR-C117977, an immunosuppressant drug that maintains long-term graft survival and induces operational tolerance, and concluded that AR-C117977 combined with CsA resulted in significant prolongation of graft survival compared with AR-C117977 or CsA therapy alone. Furthermore, CsA therapy alone did not prevent acute rejection.

Wang *et al*[28] studied local expression of B7-H4 and concluded that it prolongs islet allograft survival *in vivo*.

Studies investigating immunosuppressant drugs and their toxic effects on islets *in vivo* are still in development. The most utilized immunosuppressants were CsA, MMF and CTLA4 Ig, as shown in Table 4. The concomitant use of glucocorticoids was associated with high rejection rates and is not recommended. Their immunosuppressive and toxic effects have not been rigorously tested, and studies are still underway.

In relation to the different locations for transplantation studied according to the table, the kidney capsule was the most frequently used site for transplantation. Second was the portal vein in the liver[14]. The spleen[9,19], intraperitoneal site[32], bone marrow (tibia)[13], dorsal skin fold-intra-abdominal[30] nonmetallic chamber[11] or subcutaneous[22] was used in a small percentage of studies.

In our review of the studies, it was found that the highest survival rate was obtained by Wee *et al*[14], who used the portal vein (liver) as the site of allograft transplantation and sacrificed the mice at 100 d postoperative. Melzi *et al*[23], Watanabe *et al*[16] and Merani *et al*[10] obtained a survival rate of 100 d, where the site of engraftment

Table 5 Quantitative analysis of immunosuppressant drugs use

Ref.	Immunosuppression	Dose	Administration frequency
Fotiadis *et al*[9]	MMF and CsA	12 mg/kg and 23 mg/kg (MMF) 5 mg/kg (CsA)	-
Merani *et al*[10]	AEB-071 (Protein Kinase C Inhibitory) + CsA, CTLA4-Ig, MMF	30 mg/kg (AEB-071) 2.5 mg/kg and 5 mg/kg (CsA) 0.25 mg (CTLA4-Ig Intraperitoneal) 10 mg/kg (MMF)	2 times a day, oral (AEB-071) 2 times a day, oral (CsA) 0, 2, 4 and 6 PO, Intraperitoneal (CTLA4-Ig) Once a day, oral (MMF)
Nishimura *et al*[11]	Tacrolimus	0.5 mg/kg	Infused subcutaneously - Daily - for 14 d
Makhlouf *et al*[12]	Blockade of CD28:B7 and anti-CD40 L; CTLA-4	250μg	Intraperitoneal - 0, 2, 4 and 6 PO
Wee *et al*[14]	CsA+Tautomycetin (Synergist)	5 mg/g and 15 mg/kg (CsA)	Once a day for 7 d
Watanabe *et al*[16]	Tacrolimus and DHMEQ	1.5 mg/kg (Tacrolimus) 20 mg/kg (DHMEQ)	Once a day 0 to 3 PO and 2 times a day 0 to 14 PO (DHMEQ); 0 to 14 PO (Tacrolimus); Once a day 0 to 3 PO (DHMEQ)+0 to 14 PO (Tacrolimus)
Xekouki *et al*[18]	CsA and MMF1	5 mg/kg (CsA) 12 mg/kg (MMF) 23 mg/kg (MMF)	Oral - Daily - 12 consecutive days
Baker *et al*[19]	Monoclonal antibody antiBIP-10	300 μg intraperitoneal	Daily - 14 d[1]
Vieiro *et al*[21]	Tritiated thymidine (preoperative) and CsA	20 mg/kg (CsA)	N/A
Melzi *et al*[23]	Rapamycin + FK506 + anti–IL-2Ra chain mAbs and rapamycin+IL-10	1 mg/kg (Rapamycin) 0.05 μg/kg (IL-10) 0.3 mg/kg (FK506) 1 mg/kg (mAbs)	Intraperitoneal: Once a day - 30 PO (Rapamycin) 2 times a day - 30 d (IL-10) Once a day - 30 d (FK506) 0.4 PO (mAbs)
Fan *et al*[25]	LTβ R-Ig, CTLA4-Ig or LTR mAb anti mouse	200 μg	Intraperitoneal- days -1, 1, 3, 5, 7 and 9
Jung *et al*[26]	CD154 mAb (MR1) anti mouse + ROS-A	250 μg (CD154 mAb (MR1) anti mouse) 200 mg/kg of Ros A	Intraperitoneal injection 0, 2, 4, 6 and 8 PO (CD154 mAb (MR1) anti mouse) 8 consecutive days (ROS-A)
Påhlman *et al*[27]	AR-C117977 or CsA	0.2 mL - 3, 10, 30, or 100 mg/kg (AR-C117977) 0.5 mL - 20 mg/kg (CsA)	Subcutaneous - once a day 0 to 9 PO (AR-C117977) Once a day 0-9 PO or 0-39 PO (CsA)
Wang *et al*[28]	B7-H4	5 plaque-forming units (pfu) of Ad-B7-H4 or Ad-LacZ	N/A
Potiron *et al*[32]	CTLA4 Ig or CD40 Ig	5 10^9 IP of AdCTLA4 IM and/or 5 10^9 IM or 2 10^9 Ⅳ of AdCD40Ig; IM administration: 10 μL per point (3 points) Ⅳ administration: 150 μL with 0.9% sodium chloride	IM administration - anterior tibialis muscle; Ⅳ administration - venile vein
Jahr *et al*[33]	Anti-rat antilymphocyte serum	Intraperitoneal administration 0.5 mL	1 d after islet transplantation

[1]First dose administered 4 h preoperatively. N/A: Not available; MMF: Mycophenolate mofetil; CsA: Cyclosporine A.

Table 6 Analysis of induction and treatment of diabetic process with islet transplantation

Ref.	Number of transplanted islets	Diabetes induction method	Hyperglycemia induction (preoperative)	Normalization of hyperglycemia (postoperative)	Graft rejection	Criteria for primary graft dysfunction (PGD)
Fotiadis *et al*[9]	1812 ± 145	Streptozotocin (60 mg/kg) + PBS-Solution (Phosphate Buffer Solution) - 10 mg/mL (pH 4,5);	7 d	3 d	12 d (MMF) 10 d (CsA)	Glucose above 200 mg/dL; after 2nd PO 2 consecutive times
Merani *et al*[10]	1500	Streptozotocin (75 mg/kg) intraperitoneal	5 d	3 d	22 d	Glucose above 324 mg/dL after 2 consecutive days
Nishimura *et al*[11]	2-10/dorsal skinfold chamber	-	-	-	N/A	-
Makhlouf *et al*[12]	350 (Balb/c) 700 (NOD)	Streptozotocin and spontaneously (225 mg/kg in peritoneal cavity)	2 wk	3 d	10 d (Balb/c) 5 d (NOD) and 7 d complete rejection (NOD)	200 mg/dL - 2 to 3 consecutive days
Salazar-Bañuelos *et al*[13]	840 (of Wistar)	-	-	-	N/A[1]	N/A

Wee *et al*[14]	4000	Streptozotocin (35 mg/kg)	N/A	N/A	Untreated - 5.2 d (± 0.5) TMC - 5, 1 d (± 0, 9) TMC (0.03 mg/kg) + CsA (5 mg/kg) - > 41 d TMC (0.1 mg/kg) + CsA (5 mg/kg) - 103.8 ± 56.8 d	200 mg/dL after 2 consecutive days
Plesner *et al*[15]	550	Streptozotocin (375 mg/dL) intraperitoneal	3-5 d	5 d[1]	60 d	⩾ 198 mg/dL after 2 consecutive days
Watanabe *et al*[16]	600 or 300	Streptozotocin (180 mg/kg) intraperitoneal	5-7 d	N/A	69 d (Tacrolimus) 100 d (DHMEQ 3 d and Tacrolimus 14 d)	> 350 mg/dL for 2 consecutive days
Gysemans *et al*[17]	300	Alloxan (90 mg/kg)	24 h	N/A	N/A - 23%	Glucose level > 200 mg/dL more than 3 consecutive days
Xekouki *et al*[18]	2000	Streptozotocin (60 mg/kg) diluted in phosphated solution 10 mg/mL	1 wk	N/A	7 d (MMF ´1)	-
Baker *et al*[19]	300	Streptozotocin (220 mg/kg)	N/A	N/A	7 d	-
Li *et al*[20]	400 (200/Kidney capsule)	Streptozotocin (220 mg/kg)	N/A	N/A	8.36 ± 1.67 (islets of FVB) 16.2 ± 2.52 (islets of MT)	Glucose levels > 250 mg/dL - 2 consecutive measurements
Vieiro *et al*[21]	200	Streptozotocin (270 mg/kg) intraperitoneal	N/A	N/A	3-7 d	⩾ 250 mg/dL - 3 consecutive measurements
Neuzillet *et al*[22]	550	N/A	N/A	4 h	PEG-Solution 8 kDa 27.50 ± 3.70 d; PEG PEG-Solution 20 kDa 23.13 ± 4.39 d; PEG-Solution 35 kDa 13.80 ± 3.49 d	> 199.8 mmol/L - 2 consecutive measurements
Melzi *et al*[23]	400	175 a 200 mg/kg intravenous	1-2 wk	5 d	29 d (mouse with Glucose levels < 450 mg/dL) and 16 d (mouse with Glucose levels > 450 mg/dL)	> 250 mg/dL - 2 consecutive measurements on postoperative
Fiorina *et al*[24]	NA	Streptozotocin	N/A	N/A	14 d	N/A
Fan *et al*[25]	500	Streptozotocin (200 mg/kg)	N/A	N/A	27 d (LT[α]R-Ig) 55 d (CTLA4-Ig) After 100 d or more (LT[β]R-Ig and CTLA4-Ig)	> 300 mg/dL- after 2 consecutive days
Jung *et al*[26]	300 IEQ	Streptozotocin (180 mg/kg)	N/A	1 d	ROS-A - 53 d MR1 - 82 d ROS-A+MR1 - > 160 d	> 200 mg/dL - 2 consecutive measurements on the same week
Påhlman *et al*[27]	500-600	Alloxan Intravenous	N/A	N/A	CsA - 16 d AR-C117977, 10 mg/kg - > 100 d AR-C117977, 30 mg/kg - 29, 33 d	N/A
Wang *et al*[28]	400	Streptozotocin (200 mg/kg)	3-4 d	3 d	B7-H4 - approximately 60 d Ad-LacZ - approximately > 20 d	> 250 mg/dL after primary graft success
Chen *et al*[29]	3000 IEQ	Streptozotocin dissolved in saline (50 mg/kg)	N/A	1 wk	13 wk (SGA - microencapsulated) 7 wk (ABa-microencapsulated) 5 wk (APA-microencapsulated)	N/A

Giraud *et al*[30]	1400 IEQ	Streptozotocin (250 mg/kg), intraperitoneal	N/A	N/A	SCOT + PEG - Solution 20 kDa 310 g/L - 20 d, CMRL-1066 + 1% BSA - Solution 17.5 ± 1 d, UW-Solution - 17.2 ± 0.4 d, SCOT without PEG -14 ± 0.9 d HBSS + 0.5% BSA - Solution - 14 ± 0.7 d	> 200 mg/dL - 2 consecutive measurements
Qi *et al*[31]	1940 (± 39)	Streptozotocin (55 mg/kg)	7 d	N/A	N/A	N/A
Potiron *et al*[32]	800-1000	Streptozotocin (180 mg/kg)	1 wk	4 d	24.3 ± 9.7 d	250 mg/dL on 2 successive measurements
Jahr *et al*[33]	700-900	Streptozotocin (55 mg/kg)	7-10 d	Right after transplantation	1 wk	> 300 mg/dL after 8.9 ± 0.7 d

[1]Rodents that had normalized blood glucose in 5 d were included in the study. N/A: Not available; IEQ: Islet equivalents.

Table 7 Analysis of the parameters of hyperglycemia

Ref.	Blood Glucose Levels: hyperglycemia (mg/dL)
Fotiadis *et al*[9]	180 on 2 consecutive measurements
Merani *et al*[10]	180 on 3 consecutive days
Makhlouf *et al*[12]	Moderate diabetes: between 240 and 350 Severe diabetes: between 351 and 550 Very severe diabetes: more than 550
Wee *et al*[14]	200
Plesner *et al*[15]	⩾ 360
Watanabe *et al*[16]	200 on 2 consecutive days
Gysemans *et al*[17]	200 on 2 consecutive days
Xekouki *et al*[18]	300
Baker *et al*[19]	300 on 3 consecutive days
Li *et al*[20]	350 to 500 after 2 consecutive measurements
Melzi *et al*[23]	250 on 2 consecutive measurements
Fan *et al*[25]	300 on 2 consecutive measurements
Jung *et al*[26]	300 on 2 consecutive days
Påhlman *et al*[27]	288 on 2 consecutive measurements
Wang *et al*[28]	300 after 2 consecutive days
Chen *et al*[29]	302.4 on more than 3 consecutive days
Giraud *et al*[30]	350 after 2 consecutive days
Qi *et al*[31]	450
Potiron *et al*[32]	> 200

was the kidney capsule.

It is important to note that there was no standard level of hyperglycemia that the mice must present to be recipients of islet transplantation. A range of blood glucose levels from 180 mg/dL to 500 mg/dL was observed, as shown in Table 7.

The articles have many independent variables that influence the study results, such as species of rodent, immunosuppressant drugs and dosages, criteria for diabetes and allograft site. Thus, more research is needed to develop the ideal allograft model of islet transplantation.

CONCLUSION

Based on the analyzed studies, we can infer that islet allotransplantation in rodents is promising and continues to develop. The survival rates of allografts have increased with the discovery of new immunosuppressive drugs and the use of different graft sites. These advancements have the potential to overcome the scarcity of islet donors and improve the treatment of type 1 diabetes.

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ORIGINAL ARTICLE

Epidermal growth factor upregulates serotonin transporter and its association with visceral hypersensitivity in irritable bowel syndrome

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Abstract

AIM: To investigate the role of epidermal growth factor (EGF) in visceral hypersensitivity and its effect on the serotonin transporter (SERT).

METHODS: A rat model for visceral hypersensitivity was established by intra-colonic infusion of 0.5% acetic acid in 10-d-old Sprague-Dawley rats. The visceral sensitivity was assessed by observing the abdominal withdrawal reflex and recording electromyographic activity of the external oblique muscle in response to colorectal distension. An enzyme-linked immunosorbent assay was used to measure the EGF levels in plasma and colonic tissues. SERT mRNA expression was detected by real-time PCR while protein level was determined by Western blot. The correlation between EGF and SERT levels in colon tissues was analyzed by Pearson's correlation analysis. SERT function was examined by tritiated serotonin (5-HT) uptake experiments. Rat intestinal epithelial cells (IEC-6) were used to examine the EGF regulatory effect on SERT expression and function *via* the EGF receptor (EGFR).

RESULTS: EGF levels were significantly lower in the rats with visceral hypersensitivity as measured in plasma (2.639 ± 0.107 ng/mL *vs* 4.066 ± 0.573 ng/mL, $P < 0.01$) and in colonic tissue (3.244 ± 0.135 ng/100 mg *vs* 3.582 ± 0.197 ng/100 mg colon tissue, $P < 0.01$) compared with controls. Moreover, the EGF levels were positively correlated with SERT levels ($r = 0.820$, $P < 0.01$). EGF displayed dose- and time-dependent increased SERT gene expressions in IEC-6 cells. An EGFR kinase inhibitor inhibited the effect of EGF on SERT gene upregulation. SERT activity was enhanced following treatment with EGF (592.908 ± 31.515 fmol/min per milligram *vs* 316.789 ± 85.652 fmol/min per milligram protein, $P < 0.05$) and blocked by the EGFR kinase inhibitor in IEC-6 cells (590.274 ± 25.954 fmol/min per milligram *vs* 367.834 ± 120.307 fmol/min per milligram protein, $P < 0.05$).

CONCLUSION: A decrease in EGF levels may contribute to the formation of visceral hypersensitivity through downregulation of SERT-mediated 5-HT uptake into enterocytes.

Key words: Epidermal growth factor; Visceral hypersensitivity; Rat models; Serotonin transporter; Rat small intestinal epithelial cells; Intestinal epithelial cells; Irritable bowel syndrome

Core tip: Results of this study show that visceral hypersensitivity results in a decrease in the plasma and colon tissue levels of epidermal growth factor (EGF).

Moreover, the EGF levels were positively correlated with serotonin transporter (SERT) levels. SERT gene expression and protein activity were upregulated in a dose- and time-dependent manner by EGF, and an inhibitor of the EGF receptor kinase blocked SERT gene expression and activity in an intestinal epithelial cell line. The data suggest that decreased EGF levels may contribute to the formation of visceral hypersensitivity through downregulation of SERT activity.

Cui XF, Zhou WM, Yang Y, Zhou J, Li XL, Lin L, Zhang HJ. Epidermal growth factor upregulates serotonin transporter and its association with visceral hypersensitivity in irritable bowel syndrome. *World J Gastroenterol* 2014; 20(37): 13521-13529 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13521.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13521

INTRODUCTION

Irritable bowel syndrome (IBS), a common chronic functional gastrointestinal disease, is characterized by abdominal pain and discomfort, and bowel disturbance. The pathogenesis of IBS remains unclear; however, visceral hypersensitivity is the most likely cause for the motor and sensory abnormalities in IBS patients[1]. Recent reports indicate abnormalities in serotonergic signaling systems being involved in the development of IBS, particularly those affecting serotonin (5-HT) levels in the gastrointestinal tract[2]. Therefore, it is of interest to investigate the role of this pathway in the pathogenesis of IBS.

High levels of 5-HT have been found in the intestinal mucosal tissue of IBS patients, especially those with constipation[3]. 5-HT is known to facilitate communication between the enteric nervous system and its effector systems (muscles, secretory endothelium, endocrine cells, and vasculature of the gastrointestinal tract). An increase in 5-HT can lead to gastrointestinal motility disorder and visceral hypersensitivity[4]. Accumulating evidence suggests that alterations in serotonergic signaling exist in the gut of IBS patients, including alterations in 5-HT biosynthesis, release, and/or reuptake[5,6].

The serotonin transporter (SERT) is mainly localized to the apical membrane of intestinal epithelial cells. Due to its role in reuptake of 5-HT, SERT plays an important part in terminating transmitter action and maintaining transmitter homeostasis[7,8]. SERT gene expression is downregulated in the colon[9] and rectal tissues[10] of patients with IBS and inflammatory bowel disease. The downregulation may contribute to the pathophysiology of these gastrointestinal disorders; however, the underlying mechanisms are still not fully understood.

Previous studies have demonstrated that epidermal growth factor (EGF) upregulates the reuptake of 5-HT by increasing SERT transcription in human intestinal epithelial cells[11,12]. EGF is a 53-amino acids peptide with a variety of biologic functions. In the gut, EGF plays an important role in intestinal proliferation, differentiation, and maturation[13]. EGF affects various processes by binding to the EGF receptor (EGFR), which is expressed on the basolateral surface of both human and rat intestinal epithelial cells[14] and is associated with certain bowel diseases, such as inflammatory bowel disease[15,16]. Our preliminary findings demonstrated that plasma EGF levels were decreased in IBS patients. To date, the role of EGF in IBS patients remains unknown. Some studies report that SERT-mediated alterations of 5-HT levels in the intestinal space are related to IBS-like syndrome[17,18].

We hypothesized that EGF regulates SERT expression *via* EGFR and SERT, consequently mediating the 5-HT reuptake, which may be involved in visceral hypersensitivity. In this study, a rodent model of visceral hypersensitivity was established by a two-week colonic infusion of 0.5% acetic acid to produce persistent chronic hypersensitivity[18-20]. SERT expression was evaluated in this model, and the association of EGF levels with SERT expression was assessed. Lastly, the effect of EGF treatment on SERT expression and its function *via* EGFR in intestinal epithelial cells were investigated.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats were purchased from Beijing Vital River Laboratories Animal Technology Co., Ltd. (Beijing, China). Rats were housed with *ad libitum* food in standard rodent cages at 22 ℃ in a 12 h light-dark controlled room. All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China), and were in accordance with the guidelines of the International Association for the Study of Pain (IASP; Washington, DC, United States).

Initiation of visceral hypersensitivity

Ten-day-old male rats (n = 10) received an intracolonic infusion of 0.2 mL of 0.5% (v/v) acetic acid in saline, 2 cm from the anus. Control 10-d-old pups (n = 10) were infused with an equal volume of saline alone[19,20]. All of the experiments were conducted on 8-wk-old rats. Two parallel electrodes were implanted in the external oblique muscles of control and colonic-sensitized rats under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneal). The end of electrode was extended to the back of the necks in both groups for electromyography (EMG) recording. All postoperative rats were housed quietly for seven days in individual cages to recuperate.

Assessment of visceral sensitivity

The visceral sensitivity of the rats was assessed by observing the abdominal withdrawal reflex (AWR) and EMG activity of the external oblique muscle in response to colorectal distension (CRD), as previously described[19-21]. Briefly, the rats were anesthetized with ether, followed by insertion of a flexible balloon, attached to a graded pressure system, into the colon 8 cm from the

anus and fixed on the tail. The EMG activity of the external oblique muscle in response to CRD was measured at different pressures (20, 40, 60, and 80 mmHg). After the rats awoke, CRD was performed with 20 s of distention, followed by a complete balloon deflation for 2 min to rest between different distentions. Using a PowerLab data acquisition system (8SP; AD Instruments, Sydney, Australia) to record EMG, the area under the curve was analyzed for each EMG signal using an in-house written computer program. AWR in response to CRD was assessed by an observer without prior knowledge of the test/control groups. AWR was scored as follows: 0 = normal behavior with no response, 1 = contraction of abdominal muscles, 2 = lifting of the abdominal wall, and 3 = body arching and lifting of pelvis[20].

Evaluation of colon inflammation

After behavior testing, colon tissue specimens (4 cm proximal to the anus) were removed from rats of both groups and fixed in 10% (w/v) buffered formalin. Tissue specimens were embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin. A pathologist blindly assessed and assigned an inflammatory grade to each section.

Detection of EGF in plasma and colon tissues

EGF levels in plasma and colon tissues were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems Inc., Minneapolis, MN, United States). Following rat anesthetization, 1 mL blood samples were removed from the orbital canthus vein plexus and collected into heparin tubes followed by centrifugation at 1000 × *g* for 5 min at room temperature. The plasma (supernatant) was then collected and stored at -70 ℃ until analysis. At the same time, colon tissue samples (0.1 g) were homogenized in phosphate-buffered saline with a glass homogenizer on ice. The homogenates were then sonicated with an ultrasonic cell disrupter (New Cheese Biotech Company, NingBo, China) and centrifuged for 5 min at 5000 × *g* at 4 ℃. The supernatants were collected and stored at -70 ℃ until EGF analysis.

Cell culture

Rat intestinal epithelial cells (IEC-6) at the 10th culture passage were obtained from the American Type Culture Collection (Manassas, VA, United States) and were cultured as previously described[22]. Briefly, IEC-6 cells were grown in Dulbecco's Modified Eagle Medium (Gibco of Thermo Fisher Scientific Inc., Waltham, MA, United States), supplemented with 10% FBS (Gibco), 2 mmol/L L-glutamine, 10 mL/L antibiotic solution containing penicillin G (10000 U/mL) and streptomycin (10000 μg/mL; Gibco), and 0.01 mg/mL insulin (Sigma-Aldrich, St. Louis, MO, United States). IEC-6 cells were seeded in polystyrene plastic culture dishes (Corning Inc., Corning, NY, United States), grown for four days (37 ℃ and 5% CO_2), and used up to the 25th passage. The medium was changed every 2-3 d. All cell treatments were conducted in serum-free medium following a 1 h period of serum starvation. IEC-6 cell monolayers were treated with 0-160 ng/mL EGF (0203B16; PeproTech, Rocky Hill, NJ, United States) for 0-48 h or with 10 μmol/L EGFR specific kinase inhibitor PD153035 (Sigma-Aldrich) to block EGFR action.

Western blot detection of SERT

Intestinal tissue samples were homogenized in potency lysate buffer [25 mmol/L Tris-HCl (pH 7.5); 5 mmol/L EDTA, 5 mmol/L EGTA, 0.5 mmol/L PMSF, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L sodium vanadate] with a glass homogenizer on ice. Cells were lysed in ice-cold cell lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% (w/v) Triton X-100, 0.1% (w/v) sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 2 μg/mL leupeptin). Protein concentrations were determined by a BCA protein assay kit (#23250; Thermo Fisher Scientific Inc.). The remaining supernatant (36 μL) was combined (1:1) with 1 × sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer, and boiled for 5 min. Approximately 30 μg of protein from each sample was run on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane for immunoblotting as previously reported[23,24]. The membranes were blocked with 5% (w/v) non-fat milk, and SERT protein was detected using rabbit anti-SERT polyclonal antibody (AB9726, 1:500; EMD Millipore, Billerica, MA, United States). Mouse anti-GAPDH (Macclesfield, Cheshire, United Kingdom) polyclonal antibody (1:3000) was used as a reference. The membranes were incubated with either horseradish peroxidase-conjugated goat anti-rabbit (BS13278, 1:3000; Bioworld, Louis Park, MN, United States) or goat anti-mouse secondary antibodies (BS12478, 1:3000; Bioworld). All the antibodies were diluted with 5% (w/v) non-fat milk. The SERT/GAPDH ratio was calculated from the films with the Quantity One Analysis Software (Bio-Rad, Hercules, CA, United States), and the results were expressed in densitometric units.

Extraction of RNA and real-time PCR detection of SERT mRNA

The total RNA from control and EGF-treated cells was extracted using TRIzol reagent® (Invitrogen of Thermo Fisher Scientific Inc.). After cDNA was synthesized with a two-step reverse transcription kit (Takara, Dalian, China), real-time PCR was performed on an Applied Biosystems 7500 Real-time PCR System using the SYBR Premix Ex Taq Kit (Applied Biosystems of Thermo Fisher Scientific Inc.) in a 96-well plate. PCR cycle parameters were as follows: initial denaturation at 95 ℃ at 30 s, 40 cycles of 95 ℃ for 5 s and 60 ℃ for 34 s, followed by a dissociation stage for recording the melting curve. The cycle threshold (Ct) values of all genes were obtained, and the relative level of SERT gene was nor-

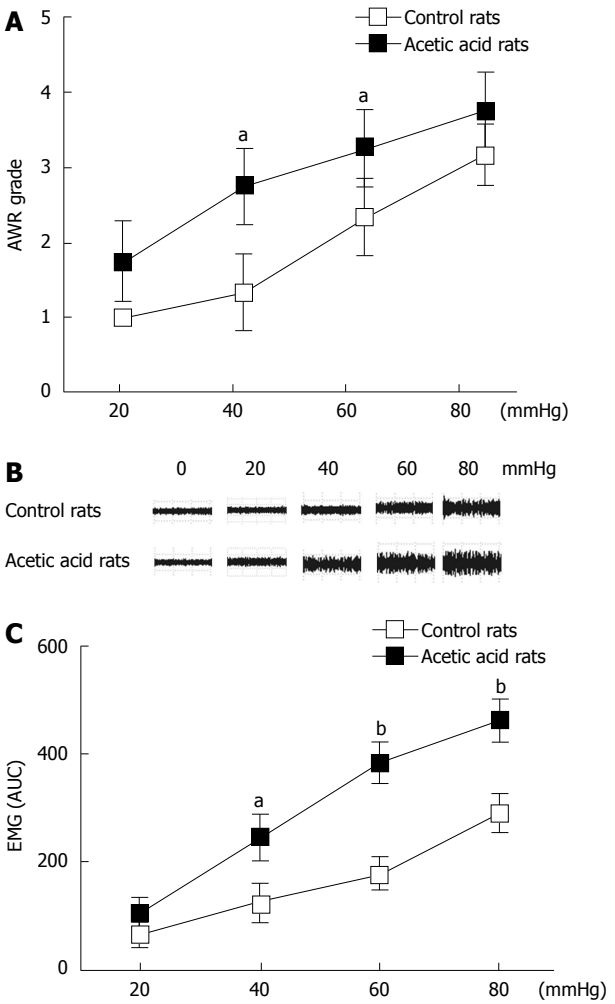

Figure 1 Effect of neonatal acetic acid treatment on 8-wk-old rat sensitivity to colorectal distension. A: Abdominal withdrawal reflex (AWR) responses to the graded pressures of colorectal distension (CRD) in saline-treated (n = 10) and acetic acid-treated (n = 10) rats. Acetic acid-treated rats show increased AWR scores compared with the saline rats. Values are expressed as mean ± SD; B: Representative electromyogram (EMG) traces recorded in control and acetic acid-treated rats in response to CRD; C: EMG responses to CRD in rats treated with saline and acetic acid at the neonatal stage. Similar to the AWR scores, acetic acid-treated rats exhibited exaggerated EMG activity responses to CRD at different pressures compared with the saline-treated rats. Neonatal rats *vs* control rats, $^{a}P < 0.05$, $^{b}P \leqslant 0.01$, error bars represent the mean ± SD.

malized to a housekeeping β-actin gene. Data were analyzed according to the relative expression using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate, and the mean values are presented. Primers used for the PCR were: forward, 5'-GACTCCTCCCCTCTAAGCCA-3' and reverse, 5'-CACGGAAAGAAGTGGTCGGA- 3' for SERT; forward, 5'-CTAAGGCCAACCGTGAAAAG-3' and reverse, 5'-TCTCAGCTGTGGTGGTGAAG-3' for β-actin.

[^{3}H]-5-HT reuptake experiment

[^{3}H]-5-HT uptake was examined as previously described[8,25]. Briefly, cells were plated in poly-D-lysine-coated (0.1 mg/mL) 12-well plates (Corning, Inc.) for 48 h before the uptake experiments. Cells (at 80%-90% confluence) were treated either with the optimal dose of EGF (40 ng/mL) in a serum-free medium or with an equal volume of the albumin-containing medium for 24 h. For the [^{3}H]-5-HT uptake assay, the growth medium was completely removed, and 1 mL of Krebs-Ringer's (KRH) buffer (130 mmol/L NaCl, 1.3 mmol/L KCl, 2.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.8 g/L glucose, 10 mmol/L HEPES, pH 7.4) was used to wash the cells twice. Next, cells were incubated in KRH buffer containing 100 μmol/L pargyline and 100 μmol/L ascorbic acid in a 5% CO_2 homothermal chamber (37 ℃) for 10 min. The uptake experiment was initiated by adding 0.1 μM [^{3}H]-5-HT (27.9 Ci/mmol, NET498; PerkinElmer, Waltham, MA, United States) and incubating the cells at 37 ℃ for another 10 min. The uptake assays were terminated by three rapid washes with cold KRH buffer. The cells were then lysed with 600 μL of 0.5 N NaOH, and 100 μL of the cell lysate was diluted in OptiPhase SuperMix scintillation mixture (Wallac, Gaithersburg, MD, United States) for direct quantification of radioactivity in a Wallac liquid scintillation counter (PerkinElmer). The total protein in the cell lysates was determined using a Bradford assay kit (Beyotime Biotechnology, Haimen, China). Specific SERT-mediated uptake was calculated by subtracting the uptake of [^{3}H]-5-HT in the presence of 100 μM paroxetine, a SERT inhibitor (Enzo, Farmingdale, NY, United States), from the total uptake.

Statistical analysis

The data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, United States). Results are expressed as mean ± SD. Two-tailed Student's t tests were used to analyze differences between two mean values. Two-way repeated measures analysis of variance (ANOVA) was used to evaluate whether the AWR scores and/or EMG area under the curves were altered in the test and/or control groups at different pressures. The remaining data were analyzed using one-way ANOVAs. Values were considered statistically significant if P was < 0.05.

RESULTS

Visceral hypersensitivity development after neonatal colonic sensitization

Neonatal rats (n = 10) treated with 0.5% acetic acid developed visceral hypersensitivity. Compared with the control rats (n = 10), the AWR scores in the acetic acid-treated rats were higher during CRD at all tested distension pressures (Figure 1A). These differences were significant at distension pressures of 40 mmHg ($P < 0.01$) and 60 mmHg ($P < 0.05$). Similar to the AWR results, acetic acid-treated rats showed increased EMG responses to CRD at three distension pressures tested (Figure 1B). The EMG area under the curve of the acetic acid-treated rats was significantly higher compared to the controls at 40 mmHg ($P < 0.05$), 60 mmHg ($P < 0.01$), and 80 mmHg ($P < 0.01$) (Figure 1C). No evidence of inflammation or structural abnormalities was found in the control or ace-

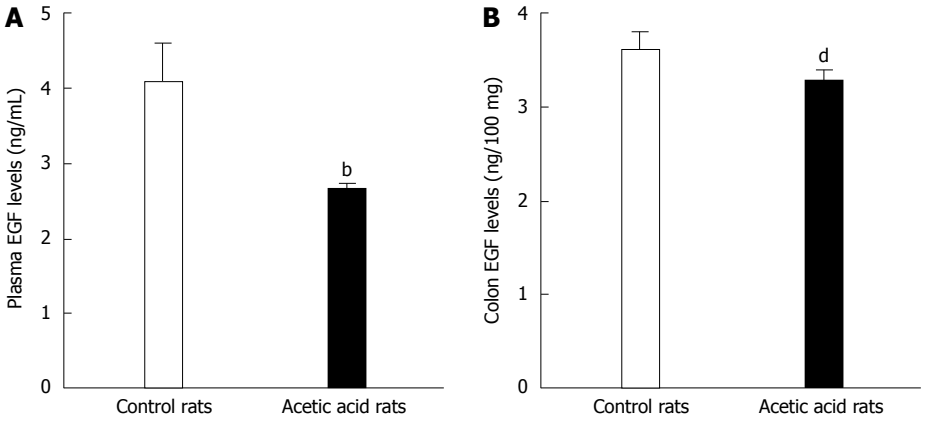

Figure 2 Epidermal growth factor levels in plasma and colon tissue of the visceral-sensitized rats. A: Plasma epidermal growth factor (EGF) levels were significantly lower in visceral-sensitized rats than in controls (visceral hypersensitive group rats *vs* control group rats, $^{b}P < 0.01$); B: EGF levels in colon were significantly lower in visceral-sensitized rats than in controls (visceral-sensitized rats *vs* control rats, $^{d}P < 0.01$). Error bars represent the mean ± SD ($n = 10$ in each group).

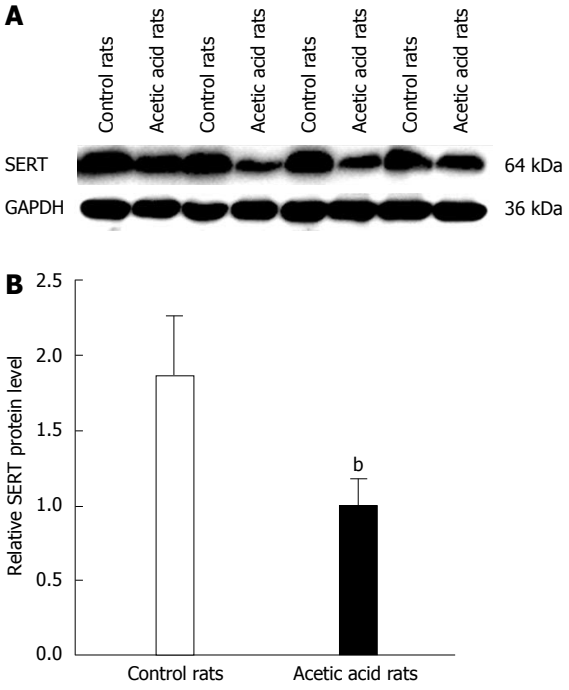

Figure 3 Serotonin transporter levels in colon tissues of control and visceral-sensitized rats. A: Western blot of serotonin transporter (SERT) expression in colonic tissues in both acetic acid- and saline-treated rat groups; B: Quantitation of SERT protein in rat colonic tissue in both acetic acid- and saline-treated rat groups compared to GAPDH. The SERT protein expressions in colon tissues were significantly lower in visceral-sensitized rats than in the controls (visceral-sensitized rats *vs* controls, $^{b}P < 0.01$) ($n = 10$ in each group).

tic acid-treated rats, indicating successful establishment of visceral hypersensitivity.

EGF levels in plasma and colon tissues were lower in rats with chronic visceral hypersensitivity

ELISA was used to detect any potential differences in EGF levels between the visceral hypersensitive and control group rats. As shown in Figure 2A, the visceral-sensitized rats had significantly lower plasma EGF levels compared with control rats (2.639 ± 0.107 ng/mL *vs* 4.066 ± 0.573 ng/mL, $P < 0.01$). The EGF levels in colon tissue from visceral-sensitized rats were also significantly decreased compared with control rats (3.244 ± 0.135 ng/100 mg *vs* 3.582 ± 0.197 ng/100 mg colon tissue, $P < 0.01$) (Figure 2B).

Expression of SERT in colon tissues decreased in rats with chronic visceral hypersensitivity

Western blot analysis showed a decrease in SERT protein levels in colon tissue of the visceral-sensitized rats (Figure 3). Analysis of the relationship between EGF and SERT levels in colon tissue showed a positive correlation ($r = 0.820$, $P < 0.01$).

Effects of EGF on SERT expression and function in intestinal epithelial cells

To observe the effect of EGF on SERT levels, IEC-6 cells were treated with various concentrations of EGF for 24 h followed by western blot analysis of SERT protein levels. Our results indicate that EGF upregulates SERT in a dose-dependent manner, with a peak at a dose of 40 ng/mL (Figure 4A). IEC-6 cells were also treated with the optimal dose of EGF (40 ng/mL) for 0, 3, 6, 12, 24, or 48 h. SERT expression was upregulated after a 12 h EGF treatment, with maximal effects after 24-48 h (Figure 4C). The real-time PCR analysis of the EGF effects on SERT gene expression showed that EGF increased SERT mRNA expression in a dose- and time-dependent manner in IEC-6 cells (Figure 4B and D).

A [^{3}H]-5-HT uptake assay was used to determine whether the high SERT expression, induced by EGF, influenced the reuptake activity of SERT in IEC-6 cells. Uptake of [^{3}H]-5-HT was significantly higher in cells pretreated with EGF (40 ng/mL) for 24 h than in the solvent controls (592.908 ± 31.515 fmol/min per milligram *vs* 316.789 ± 85.652 fmol/min per milligram protein, $P < 0.05$) (Figure 4E).

Effects of EGF on SERT gene expression and function via EGFR

To determine whether the effects of EGF on SERT ex-

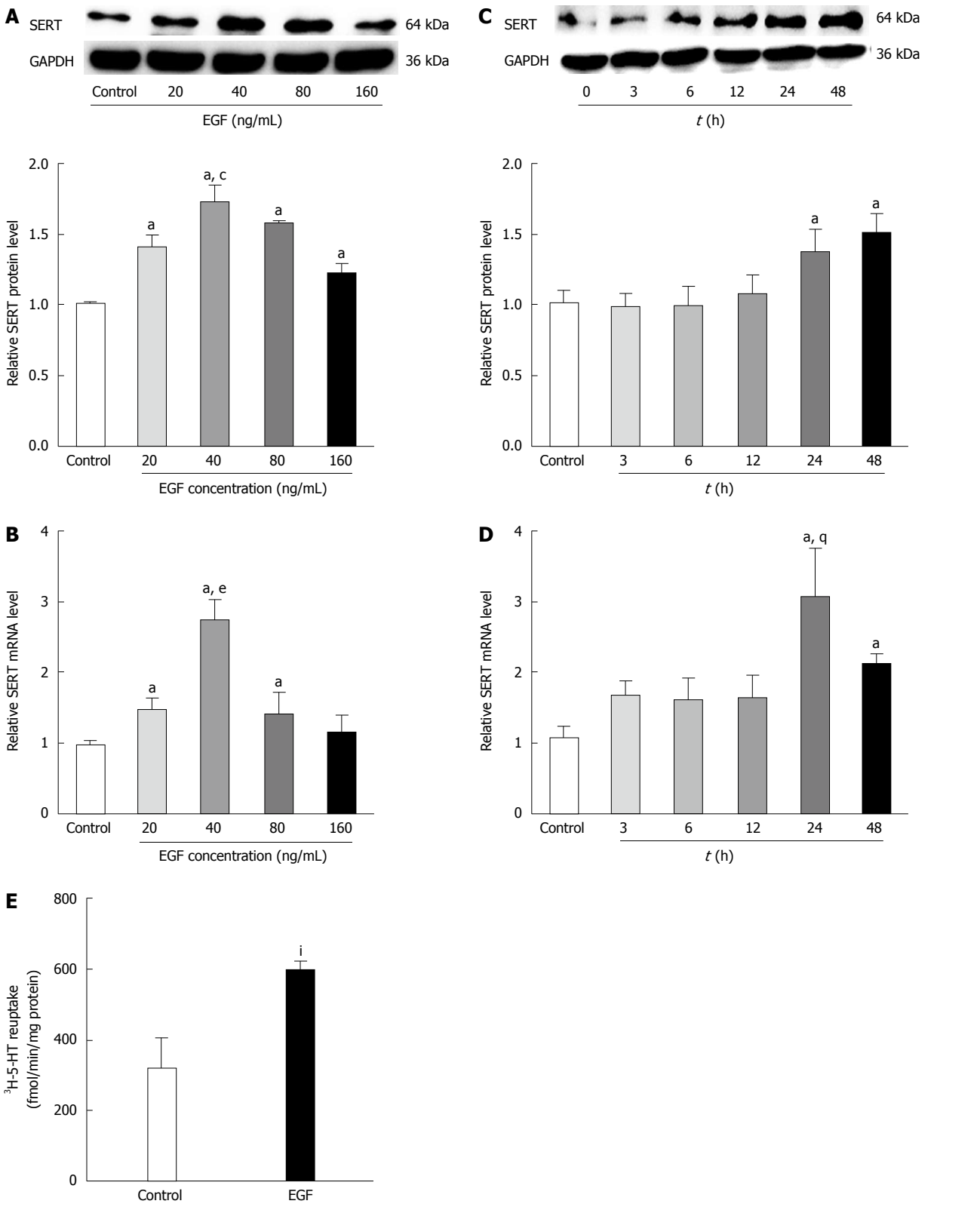

Figure 4 Effects of epidermal growth factor on serotonin transporter in rat intestinal epithelial cells. Intestinal epithelial cells (IEC-6) cells were treated with epidermal growth factor (EGF) (0, 20, 40, 60, and 80 ng/mL) for 24 h. A: Western blots were performed to detected serotonin transporter (SERT) protein expressions. GAPDH was used to verify equivalent protein loading ($^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* 20, 80, and 160 ng/mL); B: SERT gene expression was examined by real-time PCR ($^{a}P < 0.05$ *vs* control; $^{e}P < 0.05$ *vs* 20 and 80 ng/L). To determine the optimal time for EGF treatment, IEC-6 cells were treated with EGF (40 ng/L) for the indicated times (0, 3, 6, 12, 24, and 48 h); C: SERT protein levels were examined by Western blot ($^{a}P < 0.05$ *vs* control); D: *SERT* gene expression was examined by real-time PCR ($^{a}P < 0.05$ *vs* control; $^{g}P < 0.05$ *vs* 48 h); E: Uptake of [^{3}H]-serotonin in cells pre-treated with 40 ng/ml EGF for 24 h ($^{i}P < 0.05$ *vs* 24 h) . All values are mean ± SD of three independent experiments.

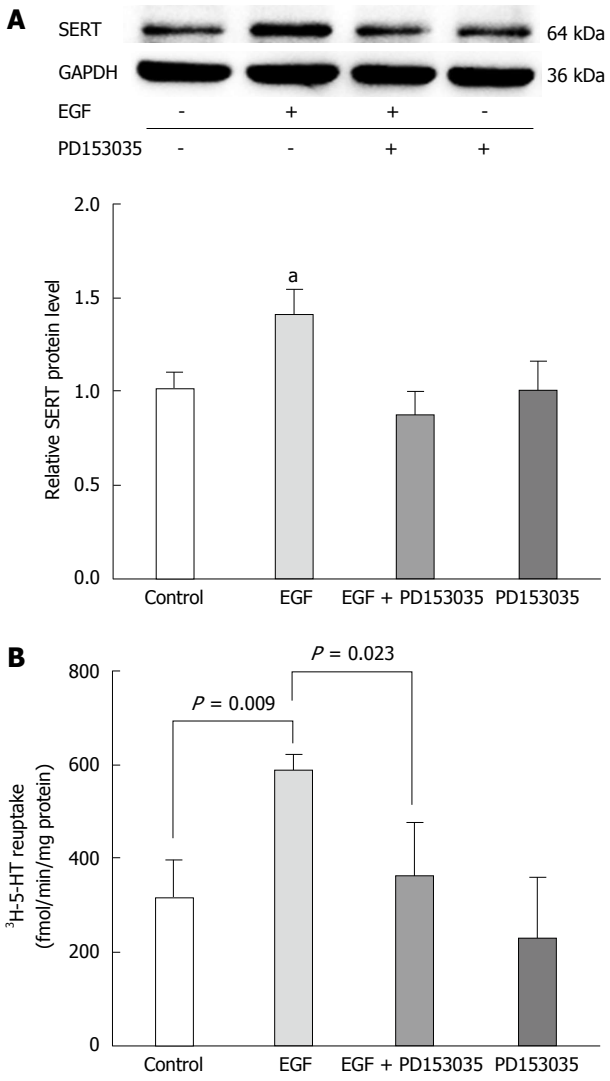

Figure 5 Role of epidermal growth factor receptor in the regulation of serotonin transporter levels and function in intestinal epithelial cells. IEC-6 cells were pre-treated with an epidermal growth factor receptor (EGFR) inhibitor (10 μmol/L PD153035) prior to stimulation with epidermal growth factor (EGF). A: Serotonin transporter (SERT) was detected by Western blot ($^aP < 0.05$ *vs* control, EGF + PD153035, and PD153035) and quantified relative to GAPDH; B: Serotonin (5-HT) reuptake was estimated by an [^{3}H]-5-HT uptake assay. Data are mean ± SD of at least three independent experiments.

pression and function were mediated *via* EGFR, IEC-6 cells were pre-treated with the EGFR kinase-specific inhibitor PD153035 (10 μmol/L) for 30 min, followed by a 24 h treatment with EGF (40 ng/mL). The results show that PD153035 blocks the EGF upregulation of SERT expression (Figure 5A), suggesting that EGF upregulates SERT expression *via* EGFR.

To investigate whether EGFR is also necessary for EGF to affect SERT reuptake activity, the [^{3}H]-5-HT uptake assay was performed in the IEC-6 cells. PD153035 (10 μM) pre-treatment abolished the EGF-induced increase in [^{3}H]-5-HT uptake in IEC-6 cells (Figure 5B), suggesting that EGF influences SERT function in 5-HT uptake *via* EGFR.

DISCUSSION

The gastrointestinal tract is a major source of endogenous 5-HT, which regulates the gastrointestinal tract's sensory, motor, and secretory functions through the interaction with different receptor subtypes. 5-HT is synthesized by tryptophan hydroxylases in enterochromaffin cells and in brainstem and myenteric plexus neurons. It is inactivated by SERT-mediated uptake into enterocytes or neurons. Recent studies have reported that patients with IBS have higher 5-HT levels and lower SERT gene expression in rectal mucosa compared with healthy controls[3,5]. These findings suggest that SERT may play an important role in the pathophysiology of IBS. In this study, SERT expression was decreased in colon tissues from visceral-sensitized rats, suggesting a potential association of SERT with sensitivity of the gastrointestinal tract.

High expression of EGF in the gastrointestinal tract[26] plays a vital role in the regulation of gastrointestinal function and protects epithelial cells from damage by various factors[27-29]. EGF has also been shown to upregulate SERT gene expression in human intestinal epithelial[11] and glial cells[30]. In our preliminary study, the plasma EGF levels in IBS patients were significantly decreased compared with healthy controls (see supplemental Figure 1). Additionally, the levels of EGF in plasma and colon tissues of visceral-sensitized rats were significantly lower than in controls. Furthermore, the Pearson's correlation analysis showed a positive correlation between EGF and SERT protein levels. These results suggest that EGF may be involved in the development of visceral hypersensitivity *via* SERT-mediated 5-HT uptake into enterocytes. Consequently, one has to ask how EGF affects SERT gene expression and function. The results of the present study show that EGF upregulates SERT mRNA expression in a dose- and time-dependent manner, suggesting regulation is at the transcriptional level. EGF-stimulated SERT gene expression is known to be dependent on tyrosine kinase activation of EGFR in human choriocarcinoma cells[31]. In this study, inhibition of EGFR blocked the effect of EGF on SERT expression and function in IEC-6 cells, thus suggesting that EGF promotes SERT-mediated 5-HT uptake into enterocytes through EGFR binding. These results are in agreement with the report by Gill *et al*[11]. EGF plays important roles in restoring tissue and enhancing SERT expression in intestinal epithelial cells; hence, promoting reuptake of 5-HT into enterocytes. Clinical studies support the beneficial effects of EGF in decreasing inflammation and diarrhea[32,33]. The beneficial effects of EGF, which may contribute to a change in visceral sensitivity, are worthy of further investigation.

In summary, plasma EGF levels were decreased both in IBS patients and in visceral-sensitized rats, and were correlated with SERT protein expression. Furthermore, treatment of cells with EGF upregulated SERT expression and function *via* EGFR. These results suggest that

EGF downregulates SERT-mediated 5-HT uptake into enterocytes, potentially contributing to the development of visceral hypersensitivity.

There are some limitations to this study. First, we did not confirm the findings concerning EGFR signaling on SERT expression and change of visceral hypersensitivity *in vivo*. Future studies are needed to use mice expressing dominant-negative EGFR point mutations to test whether the EGFR signaling pathway is involved in SERT expression and visceral hypersensitivity. Although accumulating evidence indicates that alterations in 5-HT signaling occurs in IBS, the underlying mechanism remains not well understood. Further studies are needed to better understand the pathogenesis of visceral hypersensitivity.

COMMENTS

Background

The pathogenesis of irritable bowel syndrome (IBS) remains poorly understood. Visceral hypersensitivity is the most likely culprit responsible for sensory abnormalities in IBS patients. Serotonergic signaling abnormalities are involved in the development of IBS. Serotonin transporter (SERT) gene expression is downregulated in the colon and rectal tissues of IBS patients. Epidermal growth factor (EGF) can upregulate 5-HT reuptake levels by increasing SERT expression.

Research frontiers

Visceral hypersensitivity plays an important role in the pathogenesis of IBS. However, the mechanism of visceral hypersensitivity formation remains unclear. Downregulation of SERT-mediated 5-HT uptake into enterocytes by EGF can potentially contribute to the development of visceral hypersensitivity.

Innovations and breakthroughs

There have been no studies evaluating the role that plasma EGF plays in the pathogenesis of IBS. This is the first study showing a significant decrease in the plasma level of EGF in IBS patients and rats with visceral hypersensitivity. Furthermore, SERT gene expression and protein activity were enhanced following treatment with EGF. Hence, a decrease in EGF levels may contribute to the formation of visceral hypersensitivity through downregulation of SERT-mediated 5-HT uptake into enterocytes.

Applications

The results of the study add to our understanding of EGF upregulation of SERT expression and function and its association with formation of visceral hypersensitivity. This may provide a future strategy for therapeutic intervention in the treatment of patients with IBS.

Terminology

Altered 5-HT signaling in the gut contributes to visceral hypersensitivity of IBS. Downregulated expression and function of SERT is considered a pathophysiology of various functional gastrointestinal disorders, especially IBS. EGF can increase SERT expression and function in intestinal epithelial cells, consequently decreasing 5-HT levels, thus presenting a potential therapeutic effect for visceral hypersensitivity in IBS.

Peer review

In this manuscript, the authors show that EGF levels are decreased in colon tissues and plasma of rats with visceral hypersensitivity. Furthermore, SERT expression is decreased at both protein and mRNA levels in these animals with respect to controls. The authors also show that EGF treatment induces SERT expression *via* EGFR in rat intestinal crypt cells. This research is important because it sheds light on the role that decreased EGF levels play in SERT regulation and subsequent development of visceral hypersensitivity.

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RESEARCH REPORT

Occult hepatitis B virus infection among Mexican human immunodeficiency virus-1-infected patients

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Abstract

AIM: To determine the frequency of occult hepatitis B infection (OHBI) in a group of human immunodeficiency virus (HIV)-1+/ hepatitis B surface antigen negative (HBsAg)- patients from Mexico.

METHODS: We investigated the presence of OHBI in 49 HIV-1+/HBsAg- patients. Hepatitis B virus (HBV) DNA was analyzed using nested PCR to amplify the Core (C) region and by real-time PCR to amplify a region of the S and X genes. The possible associations between the variables and OHBI were investigated using Pearson's χ^2 and/or Fisher's exact test.

RESULTS: We found that the frequency of OHBI was 49% among the group of 49 HIV-1+/HBsAg- patients studied. The presence of OHBI was significantly associated with the HIV-1 RNA viral load [odds ratio (OR) = 8.75; P = 0.001; 95%CI: 2.26-33.79] and with HIV-antiretroviral treatment with drugs that interfere with HBV replication (lamivudine, tenofovir or emtricitabine) (OR = 0.25; P = 0.05; 95%CI: 0.08-1.05).

CONCLUSION: The OHBI frequency is high among 49 Mexican HIV-1+/HBsAg- patients and it was more frequent in patients with detectable HIV RNA, and less frequent in patients who are undergoing HIV-ARV treatment with drugs active against HBV.

Key words: Hepatitis B virus; Occult hepatitis B virus infection; Human immunodeficiency virus; Hepatitis B surface antigen negative; Risk factors; Molecular diagnostics

Core tip: In this study we assessed the frequency of occult hepatitis B infection (OHBI) in a group of 49 human immunodeficiency virus (HIV-1)+/ hepatitis B surface antigen negative (HBsAg)- Mexican patients using a highly sensitive in-house core-nested PCR and real-time PCR assays for hepatitis B virus (HBV) DNA detection. In this study we showed that the frequency of OHBI is high among HIV+/HBsAg- patients

and suggests the need for testing HBV DNA in bigger populations utilizing more sentitive assays. Then, we propose to utilize the core-nested PCR assay in the initial screening for OHBI. Prospective studies would be needed to assess the clinical value of this diagnostic approach.

Alvarez-Muñoz MT, Maldonado-Rodriguez A, Rojas-Montes O, Torres-Ibarra R, Gutierrez-Escolano F, Vazquez-Rosales G, Gomez A, Muñoz O, Torres J, Lira R. Occult hepatitis B virus infection among Mexican human immunodeficiency virus-1-infected patients. *World J Gastroenterol* 2014; 20(37): 13530-13537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13530.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13530

INTRODUCTION

Occult hepatitis B infection (OHBI) is characterized by the long-lasting persistence of hepatitis B virus (HBV) DNA in the liver, with detectable or undetectable HBV DNA in the serum, in the absence of HBV surface antigen (HBsAg)[1-3]. The development of more sensitive detection systems for HBV DNA has improved the ability to detect this complex entity[3]. Although OHBI has been associated with the presence of anti-HBV antibodies directed against the viral core antigen (HBc) and/or against HBsAg, a significant proportion of individuals are negative for all HBV serum markers[4,5]. Thus, patients with OHBI can be classified as seropositive (anti-HBc and/or anti-HBs+) or seronegative (anti-HBc and anti-HBs negative)[3]. Prevalence rates for OHBI ranging from < 1% to as high as 87% have been reported from different areas around the world[6-8]. It has been reported that OHBI seems to be more prevalent among subjects at high risk for HBV infection and with concomitant liver disease[9]. Human immunodeficiency virus (HIV)-infected patients are also at risk of acquiring viral hepatitis due to common routes of transmission. Several studies have investigated the prevalence and clinical impact of OHBI in HIV-1-infected individuals, and these studies have shown that co-infection is increasing worldwide[10]. However, the clinical significance of OHBI infection in HIV-infected patients remains unclear. In HIV+/HBsAg-/anti-HBc+ patients, the prevalence of serum HBV DNA is highly variable. For example, a group of injection drug users (IDUs)[11] have no detectable levels of HBV DNA, whereas in other patients, the prevalence of HBV DNA can be as high as 89.5%[12]. The discrepancies in the reported prevalence of OHBI in HIV-infected patients vary depending on the definition used for OHBI, the sensitivity of the assay and the cutoff value for the HBV viral load[13-15]. According to the Taormina statements, the gold standard for OHBI testing is the analysis of HBV DNA in extracts from liver tissues using highly sensitive and specific techniques (nested PCR or real-time PCR) with specific primers for different HBV genomic regions and with highly conserved nucleotide sequences[16]. The aim of this study was to assess the prevalence of OHBI in group of HIV-1+/HBsAg- Mexican patients, Possible associations between the presence of HBV DNA in plasma and antibodies against HBV antigens, the HIV-1 viral load (VL), and anti-retroviral treatment (ARV) that includes drugs active against HBV replication [lamivudine (3TC), tenofovir (TDF) or a combination of TDF/emtricitabine (FTC)] were investigated.

MATERIALS AND METHODS

Patients

Plasma samples were collected from 42 HIV-1-infected patients who were being treated at the La Raza Infectious Diseases Hospital, which is a tertiary referral center of the Instituto Mexicano del Seguro Social (IMSS), and from 13 patients attending Hospital Regional-72. A total samples of 55 were collected during the period of 2009-2011. The study protocol was approved by the Ethical Committee of the IMSS, and written informed consent was obtained from each participant.

HBV serological markers and determination of HIV RNA-1 viral load

An 8 mL sample of blood was drawn from each of the 55 patients with known diagnoses of HIV-1 infection. All serum samples were screened to detect the following HBV serological markers using commercial ELISA assays (Abbot Diagnostics Laboratories): HBsAg, anti-HBc (IgG), anti-HBs and HBeAg. Six of the 55 patients were co-infected with HBV (HBsAg+) and were eliminated from the study. A total of 49 samples were included for detection of OHBI. The HIV RNA viral load was determined in plasma using the Cobas Amplicor RNA HIV-1 Monitor™ test *vs* 1.5 (Roche Diagnostics, IN, USA), which has a known sensitivity of 50 copies/mL.

Diagnosis of OHBI infection by amplification of three HBV genome regions

According to Raimondo[2], PCR primers should be designed to span at least three genomic regions of the HBV genome to avoid false-negative or false-positive results. In this study two different approaches, a higly nested PCR assay and a quantitative qPCR with syber green were utilized in order to identify the HBV DNA in the occult HBV coinfected individuals, and confirm the utility of a simple nested assay to screen the presence of very low concentrations of HBV DNA. We tested for HBV DNA using assays specific for three HBV genomic regions, core (C), X and surface (S). To amplify the C region, we used a nested-PCR, whereas for the X and S regions, we designed primers for real-time PCR assays (qPCR).

Definition of OHBI: We considered a patient to be OHBI+ when his or her sample was positive for at least two different viral genomic regions or when positive for the C region and confirmed by sequencing.

RT-PCR for X and S genomic regions

Nucleic acids were extracted from plasma using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer' s instructions. The selected X and S genomic regions were amplified using real-time quantitative PCR (qPCR) utilizing SYBR Green technology in a thermal cycler LightCycler-480 (LC480) (Roche Diagnostics, Mannheim, Germany). The following specific primers were designed to specifically amplify a 277 bp fragment of the X-ORF region (nt 1601-1877): forward HBVOX-1 (5´-ACGTCGCATGGAGACCACCG-3´) and reverse HBVOX-2 (5´-GCTTGGAGGCTTGAACAGTGGGA-3´). For the S-ORF, a 145 bp fragment (nt 251-396) was amplified with the following primers: forward HBVV-Lup 5'-GACTCGTGGTGGACTTCTCTCA-3' and reverse HBV-Ldw (5´-TAAAACGCCGCAGACACATC-3'. The standard curves were calibrated with a sample known to be positive for HBV (HBV-167; VL =4 × 10^6 IU/mL), with a range of detection from 50 IU/mL to 1 × 10^5 IU/mL. Amplifications of the S- and X-ORF were carried out in the Thermal Block cycler LC480 in a 10 μL reaction volume, mixing 4 μL DNA and 6 μL of the master mix LightCycler 480 SYBRGreen I (Roche) containing forward and reverse primers. The samples were run in triplicate, and each test was repeated at least two times. Negative controls, lacking nucleic acid, were included in each run. The amplification reactions for ORF-X were performed as follows: 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 10 s, 60 ℃ for 10 s and 72 ℃ for 20 s. Amplification of the S region utilized the same conditions except for the annealing temperature, which was 58 ℃. Melting curves were obtained and threshold cycles values (Ct) were calculated with the LC480 Basic software V. 1.2 (Roche Diagnostics).

Nested PCR assay for the HBV-core

A known HBV-positive plasma sample (HBV-84), with a VL of 1330 IU/mL, was used as a positive control for the C-region nested PCR assay as previously described[17]. The limit of detection for the C-nested PCR assay was approximately 5.7 IU/mL (30 copies/mL)[17]. The outer primers that amplify a 560 bp fragment were as follows: sense (5'-TTCAAGCCTCCAAGCTGTGCCTTGG-3', nt 1863 to 1887) and antisense (5'-TCTGCGACGCGGCGATTGAGA-3', nt 2402 to 2422). The inner primers that amplify a fragment of 438 bp were as follows: sense (5'-CCTTGGGTGGCTTTGGGGCA-3', nt 1882-1901) and antisense (5'-AGGATAGGGGCATTTGGTGGTCTATA-3', nt 2294-2319). The first amplification was performed in a volume of 25 μL, containing Kappa biosystems 1 × PCR buffer, 0.5 μmol/L of each primer, 0.2 mmol/L dNTP mix; 1.25 U Kappa Taq DNA polymerase (Kapa Biosystems) and 5 μL of DNA. The reaction was run in a DNA thermal cycler (Biometra GmbH, Germany) with the following conditions for both PCR rounds: 95 ℃ for 5 min, followed by 40 cycles at 95 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 1 min with a final extension at 72 ℃ for 10 min. The second PCR round was done under the same conditions with 5 μL of the first round product. The PCR products were analyzed by electrophoresis on 1% agarose gels. PCR fragments were obtained using the gel purification extraction kit (QIAGEN GMBH, Germany) according to the manufacturer's instructions.

Sequencing

PCR products were directly sequenced in both directions with corresponding amplification primers. DNA sequencing was performed using the dideoxy method in a BigDye Terminator Cycle Sequencing Ready reaction V.2 using the ABI Prism 3100 (16-capillary) Genetic Analyzers (Applied Biosystems Foster City CA) sequencing kit reaction mix, which contains 40 ng of purified PCR product, according to the manufacturer's instructions. Sequence data were analyzed using the CLCbio software, and HBV genotypes were determined with the genotyping tool program[18].

Statistical analyses

Comparison of the discrete variables age (< 45 or ⩾ 45 years old), gender, CD4 cell count (< 200 or ⩾ 200 cell/mL), plasma HIV RNA VL (detectable: > 50 copies/mL, or not), antiretroviral (ARV) patient treatment with or without anti HBV-activity (3TC, TDF or FTC) and positivity for HBV DNA (OHBI+ and OHBI- groups) was performed using Pearson's χ^2 test or Fisher's exact test, as required. Proportions were described together with 95% confidence interval (95%CI). All data with appropriate *P* values ⩽ 0.05 were considered to be statistically significant. All the analyses were performed using SPSS for windows V.12.0 (SPSS, Chicago IL).

RESULTS

Demographic and clinical characteristics of patients

The characteristics of the 49 HIV-1+/HBsAg- patients included in the study are summarized in Table 1. The age ranged from 18 to 73 years with an average age of 45 years. The range of the HIV RNA-1 VL was from non-detectable (ND) to 5.1 $\log_{10}$ HIV-RNA copies/mL. The CD4-T cell counts varied from 70 to 1500 cells/mm. Forty-eight patients were receiving antiviral treatment (ARV), and 33 patients were treated with drugs that exhibited anti-HBV activity: 3TC, TDF, or a combination of TDF/emtricitabine (FTC). According to the HBV serological pattern of OHBI, two groups of HIV-1+/HBsAg- patients were identified: a seropositive (SP) group that included 27 patients anti-HBc+ and a seronegative (SN) group with 22 patients.

Variables associated with OHBI

Twenty-four patients (49%) were positive for OHBI, and a detailed description of genome regions amplified, the presence of serologic markers, the HIV RNA VL, and the anti-viral treatment in each of these 24 patients

Table 1 Overall demographic, serological, and clinical data of the study population

HIV-1 infected patients	*n* = 55	95%CI (%)
Ratio M:F	3:1	
Age range (yr)	18-73	
HIV/HBV coinfection (HBsAg+)	6	10.7 (5.0-21.4)
HIV-1 patients included (HbsAg-)	49	89.0 (78.2-94.9)
Seropositive OHBI (SP)	27	49.0 (36.4-61.9)
Seronegative OHBI (SN)	22	40.0 (28.1-53.2)
Range of HIV RNA viral load in plasma (log_{10} copies/mL)	ND-5.1	
CD4+ cell/mm^3	70-1500	

HIV: Human immunodeficiency virus; HBV: hepatitis B virus; SP: One or more HBV serological marker except HbsAg (anti-HBc, anti-HBe, anti-HBs); SN: Negative for all HBV markers; OHBI: Occult hepatitis B infection; ND: No detectable by Cobas Monitor Amplicor Roche V. 1.5.

is presented in Table 2. OHBI was identified in 48.1% (13) of the SP group and 50% of the SN group (11). The sequences of the HBV-core amplied fragment were analyzed using the maximum likelihood method based on the Kimura 2-parameter model and compared to HBsAg+/HIV-1 samples and genotype H reference sequence, because the high homology in the sequences to genotype H (Figure 1).

No significant differences in gender, age, CD4+ levels, and the presence or absence of HBV serologic markers were found between OHBI+ patients and OHBI- patients. A positive correlation was found between the presence of OHBI and the HIV RNA-1 VL (OR = 8.75; *P* = 0.001; 95%CI: 2.26-33.79). ARV treatment with anti-HBV activity was also significantly associated with OHBI (OR = 0.25; *P* = 0.05; 95%CI: 0.08-1.05) (Table 3).

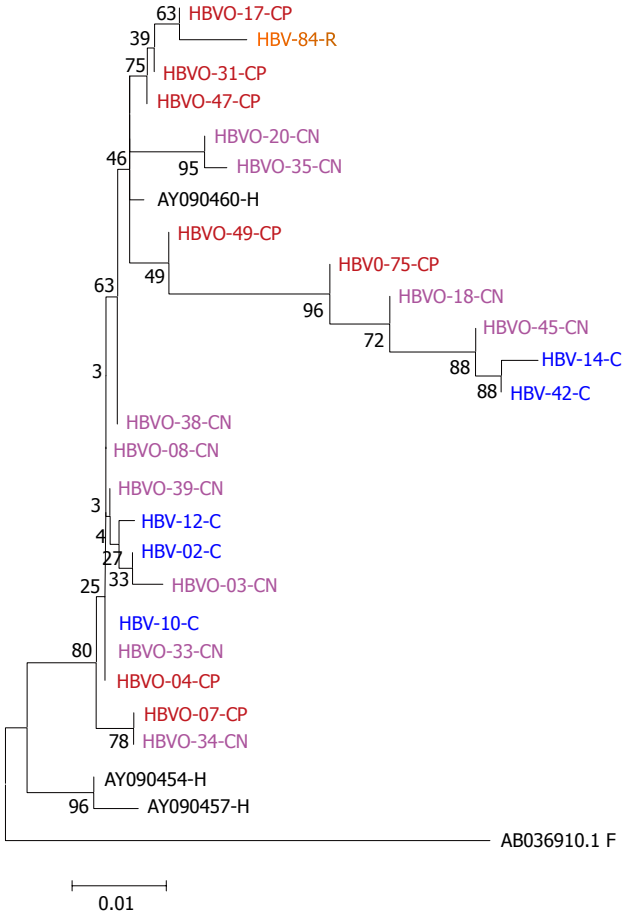

Figure 1 Phylogenetic tree constructed by the Maximum Likelihood method based on the partial nucleotide sequence of the core gen (439 bp) of 18 OHBI isolates (CP and CN); 5 sequences (C) obtained from hepatitis B virus overt co-infection (HbsAg+), using the three genotype H reference sequences from Genbank (AB375159.1, AB375160.1, AB375161.1), as a reference group and one hepatitis B virus sequence from a chronic hepatitis B virus mono-infected patient (HBV-84-R). Genetic distances were estimated using the Kimura two-parameter matrix and Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 replicates. C: Core; -CN: Seronegative OHBI; -CP: Seropositive OHBI; -S: HBsAg+ isolates.

DISCUSSION

In Mexico HBV infection endemicity is characterized by a low HBsAg seroprevalence (0.03%), apparently due to a rapid resolution of the infection, low viral loads and a high prevalence of occult Hepatitis B infection[19]. However, high endemic areas of HBV infection have been detected in native population using anti-HBc marker and molecular diagnosis of HBV genomes[20,21]. The present study reports a high prevalence (49%) of occult hepatitis B virus infection in a group 49 HIV-1-infected individuals in Mexico. Previous reports on the prevalence of OHBI in HIV-1 patients have shown controversial results, ranging from 0 to 89.5%. For example, Núñez *et al*[11] did not detect HBV DNA in HIV-positive injection drug users, whereas a prevalence of 0.8% was reported in French HIV-infected patients[22]. Additional reports from Brazil[23], and the Netherlands[24] found OHBI in 5% of HIV-infected patients. In contrast, prevalences of 14% in the UK[25] as high as 89.5% in Switzerland[12] and 88.4% in South Africa[13] have been reported. The discrepancies in the rate of OHBI may reflect the varying prevalences of HBV and HIV infections in different countries and the sensitivity of the assays used to detect HBV DNA. The prevalence of OHBI generally correlates with the level of HBV endemicity in different geographic areas. Thus, in regions with a low prevalence of HBV infection, like our country, OHBI would be high only in groups with a risk factor such as HIV-1 infection.

The C nested-PCR assay used in this study for HBV DNA detection improved our ability to detect OHBI because we had two cases negative for the X and S region but positive for nested-C PCR assay. In these cases, the identity of the OHBI circulating in the patient was confirmed by sequencing. One limitation of this study was the inability to sequence all the positive samples; we were unable to obtain the sequence in 7 of the 24 positive samples, because the amount of amplicon was very low. However, from the samples that were sequenced it is important to emphasize that they belong to genotype H. In Mexico, HBV genotype H is predominant during chronic infections and it is detected in mixtures with other genotypes and associated with other comorbities such

Table 2 Detection of hepatitis B virus DNA regions, serologic markers, and ART treatment in 24 human immunodeficiency virus-1 patients identified with occult hepatitis B infection

Patient ID	HBV serological markers			qPCR[1]		C-nested PCR	RNA HIV (CV)	HAART Tx	HBV Tx[2]
	αHBc	αHBs	αHBe	S	X		Log_{10} c/mm^3		
SP									
HBVO-04	+	-	-	+	+	+	3.1	+	-
HBVO-05	+	-	-	+	+	+	3.2	+	3TC
HBVO-07	+	+	-	-	-	+	ND	+	-
HBVO-17	+	-	+	+	+	+	2.4	+	TDF
HBVO-22	+	+	-	+	+	+	ND	+	3TC
HBVO-27	+	+	+	+	+	+	ND	+	TDF/FTC
HBVO-31	+	+	+	+	+	+	4.3	+	3TC
HBVO-32	+	+	+	+	+	+	2.2	+	3TC
HBVO-36	+	-	-	+	+	+	4.3	+	-
HBVO-47	+	-	+	+	+	+	5.1	-	-
HBVO-49	+	+	+	+	+	+	ND	+	-
HBVO-54	+	+	+	+	+	+	3.6	+	TDF/FTC
HBVO-75	+	+	+	+	+	+	ND	+	TDF/FTC
SN									
HBVO-03	-	-	-	+	+	+	5	O	-
HBVO-08	-	-	-	-	-	+	1.7	+	3TC
HBVO-18	-	-	-	+	+	+	2.6	+	-
HBVO-20	-	-	-	+	+	+	3.3	+	3TC
HBVO-26	-	-	-	+	+	+	3.9	+	-
HBVO-33	-	-	-	+	-	+	3	+	TDF
HBVO-34	-	-	-	+	+	+	2.7	+	-
HBVO-35	-	-	-	+	-	+	2.4	+	-
HBVO-38	-	-	-	+	-	+	ND	+	-
HBVO-39	-	-	-	+	+	+	ND	+	-
HBVO-45	-	-	-	+	+	+	ND	+	TDF
Frequency (%)						49			

[1]qPCR: Real time PCR utilizing SyberGreen assay; [2]ARV treatment included tenofovir (TDF). HIV: Human immunodeficiency virus; HBV: hepatitis B virus; SP: Seropositive (one or more HBV serological marker: anti-HBc, anti-HBe, anti-HBs); SN: Negative for all HBV markers; ND: No detectable; S: Surface ORF; X: Protein X ORF; C: Core ORF; Tx: Treatment; TDF/FTC: Tenofovir/emtricitabine; 3TC: Lamivudine; O: Other ARV treatment (IFN).

a coinfection with HCV or HIV-1[19]. In particular, OHBI has been identified in 6.4% of blood donors, whereas genotype H was detected in 66.7% of the samples[17]. However, there is only one previous report of 18.4% OHBI in a group of HIV-1 Mexican patients[26].

Several studies have examined the prevalence of OHBI in HIV-1 patients, and few have evaluated risk factors. We analyzed the correlations between diverse variables and OHBI. We found no association between age, sex, ARV treatment duration, or CD4-cell count and OHBI. In our study, the distribution of positive cases for HBV serologic markers was similar in both SP and SN patients. This finding is relevant because previous studies have suggested that OHBI is more prevalent in SP individuals with anti-HBc[4,27]. Some studies have reported this in 13.6% of Iranian HIV-positive patients[28], compared to 24.5% of Indian[29] and 28.7% of Lebanese HIV-infected patients[30] although other studies have reported OHBI in SN HIV-1 patients[29,31]. In contrast, we found a significant inverse association (OR = 0.25; P = 0.05; 95%CI: 0.08-1.05) in patients treated with HBV-active drugs (3TC, TDF, or FTC) and OHBI, which would suggest a protective role for OHBI prevention. This agrees with a previous report in which the use of ARV treatment with anti-HBV activity decreased the risk for OHBI[7]. However, other studies have found no correlation between 3TC treatment and OHBI[32-34]. Although the P value was at the limits of statistical significance (P = 0.05) and the statistical power of the size sample is only of 48%, the data could suggest a protective role for prevention of OHBI. These results suggest the necessity to perform the screening for OHBI in a larger group of HIV-1 patients.

Other studies have also shown that OHBI was more frequently detected in individuals with a low CD4+ cell count[24,35], an association that was not found in our study. We found that a detectable level of HIV RNA was a significant risk factor for OHBI in the group of patients studied (OR = 8.75), suggesting that an immune dysfunction other than a low CD4 cell count might impair the elimination of HBV and allow low levels of replication. In accordance with this, a study reported that occult HBV was associated with an HIV RNA level > 1000 copies/mL but not with CD4 cell counts < 200 cells/mm^3[7]. However, other studies have not found a significant association with HIV-RNA levels[22,25,36,37].

Several patients in the SN group (8/18) exhibit the coexistence of anti-HBs with HBV DNA detectable in plasma (Table 2), this prevalence is high and it would be interesting in the future to define by additional sequencing the presence of mutations at "a" major determinant at S gene, as potential mutants to escape neutralizing antibodies.

Table 3 Analysis of potential risk variables associated with hepatitis B virus infection in human immunodeficiency virus-1 patients

	HBV DNA					
	Positive (n = 24) n (%)	Negative (n = 25) n (%)	χ^2	P value	OR	95%CI
Gender						
Male	17 (71)	20 (80)	0.55	0.45	0.60	0.16-2.26
Female	7 (29)	5 (20)				
Age						
< 45 yr	12 (50)	12 (48)	0.02	0.88	0.92	0.30-2.83
⩾ 45 yr	12 (50)	13 (52)				
Seropositive for any marker (SP)						
Yes	13 (54)	14 (56)	0.01	0.89	0.92	0.30-2.86
No	11 (46)	11 (44)				
HIV RNA VL > 50 copies/mL						
Detectable	15 (63)	4 (16)	11.15	0.001	8.75	2.26-33.79
No detectable	9 (37)	21 (84)				
ARV-treatment						
Yes	24 (96)	23 (96)	-	1.0[1]	1.04	0.06-17.68
No	1 (4)	1 (4)				
With TDF/FTC or 3TC						
Yes	13 (54)	20 (80)	3.72	0.05[2]	0.25	0.08-1.05
No	11 (46)	5 (20)				
CD4+						
< 200 cell/mm^3	2 (8)	3 (12)	-	1.0[1]	0.66	0.10-4.38
> 200 cell/mm^3	22 (92)	22 (88)				

[1]Fisher's exact test; [2]α = 95%, β = 80%, OR = 0.29 with the same percentages.

The results of this study suggest that HIV+/HBsAg- patients should be tested for OHBI, particularly those with detectable levels of HIV RNA, regardless of the presence of HBV serological markers. Our results also suggest that the C-nested PCR amplification could be used for the initial screening for OHBI. Furthermore, this approach would make routine serological testing unnecessary, significantly reducing the cost of HBV diagnosis. Prospective studies would be needed to assess the clinical value of this diagnostic approach.

Our study had some limitations. The sample size was relatively small and it was a single time point testing without any follow-up. Secondly, since it was a cross-sectional study, liver and clinical hepatic characteristics other than CD4 and ARV treatment were not determined in our study.

In conclusion, this study shows that the prevalence of OHBI is high among 49 HIV+/HBsAg- patients in Mexico and suggests the need for a sensitive test for HBV DNA detection. We suggest that C-nested PCR assay might be useful in the initial screening for OHBI especially in absence of HBsAg marker.

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COMMENTS

Background

The presence of hepatitis B virus (HBV) DNA in individuals hepatitis B surface antigen negative (HBsAg)-negative is defined as occult HBV infection (OHBI). Human immunodeficiency virus (HIV)-infected patients are at risk of acquiring viral hepatitis, due to common routes of transmission. Current evidences have shown that is relatively frequent in HIV-patients, however there are conflicting reports on the impact of OHBI on the natural history of HIV disease. In Mexico, few studies have been perfomed on OHBI prevalence. More studies are needed to determine the frequency of infection in this group HIV patients should be screened for evidence of occult hepatitis B infection.

Research frontiers

The clinical significance of OHBI is not defined yet. However, this infection has been involved in different clinical context, including acute reactivation when an immunosuppressive status occurs, and there are also evidence suggesting that it may contribute to the development of cirrhosis and may have an important role in hepatocarcinogenesis.

Innovations and breakthroughs

The frequency of OHBI in HIV-1 patients is now well recognized due to advances in molecular. This is the first study in Mexico, that demonstrates the frequency of OHBI in HIV-patients utilizing more than one genetic marker in the analysis. The data presented here is a valuable contribution in the area of clinical virology because HIV-1 infection in our country is a very important health problem. Other problem is the low amount of HBV DNA that can be detected in the serum, of coinfected patients, for that more sensitive assay must be developed. The data clearly shows that the determination of the HBsAg solely may underestimate the actual prevalence of HBVO in this group of patients.

Applications

The results of this study suggest that HIV/OHBI coinfected patients remain undiagnosed, if only conventional serological markers for HBV are used. The authors propose that C-nested PCR assay might be useful in the initial screening for HBV DNA detection in bigger population groups, this approach will allow us to screen a bigger population.

Peer review

The author investigated the presence of OHBI in 49 HIV-1/HBsAg- patients. They would like to determine the frequency of OHBI in HIV-1+/HBsAg- patients in Mexico. They used nested PCR or real time PCR to amplify a region of the C, S and X genes. They found that the OHBI frequency is high among Mexican HIV-1+/HBsAg- patients and is more frequency in patients with detectable HIV RNA. The findings in this report are very important for managing HIV-1+/HBsAg patients. The study population is small, but the results are sufficient to perhaps merit an exploration in a bigger population.

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RETROSPECTIVE STUDY

Revisiting the role of pathological analysis in transarterial chemoembolization-treated hepatocellular carcinoma after transplantation

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Abstract

AIM: To define the histopathological features predictive of post-transplant hepatocellular carcinoma (HCC) recurrence after transarterial chemoembolization, applicable for recipient risk stratification.

METHODS: We retrospectively reviewed the specimens of all suspicious nodules (total 275) from 101 consecutive liver transplant recipients which came to our Pathology Unit over a 6-year period. All nodules were sampled and analyzed, and follow-up data were collected. We finally considered 11 histological variables for each patient: total number of nodules, number of viable nodules, size of the major nodule, size of the major viable nodule, occurrence of microscopic vascular invasion, maximum Edmondson's grade, clear cell/sarcomatous changes, and the residual neoplastic volume. Survival data were computed by means of the Kaplan-Meier procedure and analyzed by means of the Cox proportional hazards model. The multivariate linear regression and a k-means cluster analysis were also used in order to compute the standardized histological score.

RESULTS: The total number of nodules, the residual neoplastic volume (the total volume of all evaluated nodules minus the necrotic portion) and the microvascular invasion entered the Cox multivariate hazard model with HCC recurrence as dependent variable. The histological score was therefore computed and a cluster analysis sorted recipients into 3 risk groups, with 3.3%, 18.5% and 53.8% respectively of tumor recurrence rates and 1.6%, 11.1% and 38.5% of tumor-related mortality respectively at the end of follow-up.

CONCLUSION: The histological score allows a reliable stratification of HCC recurrence risk, especially in those recipients found out to be beyond the Milan criteria after orthotopic liver transplantation (OLT).

Key words: Chemoembolization; Hepatocellular carcinoma; Histopathology; Orthotopic liver transplantation; Cancer recurrence; Transarterial chemoembolization

Core tip: Transarterial chemoembolization (TACE) of hepatocellular carcinoma (HCC) is used for down-staging before orthotopic liver transplantation (OLT), and as "bridging therapy" to reduce the drop-out rates in the waiting list. A discrepancy exists between the pre-transplant (radiological) and post-transplant (pathological) staging. Few study analyzing the histology of TACE-treated HCC exist. Here we analyzed 11 histological variables in 275 nodules from 101 consecutive OLT recipients and we computed a histological score to stratify recipients into 3 risk groups, with 3.3%, 18.5% and 53.8% respectively of tumor recurrence rates. The histological score allows a better stratification of those recipients beyond the Milan criteria after OLT.

Vasuri F, Malvi D, Rosini F, Baldin P, Fiorentino M, Paccapelo A, Ercolani G, Pinna AD, Golfieri R, Morselli-Labate AM, Grigioni WF, D'Errico-Grigioni A. Revisiting the role of pathological analysis in transarterial chemoembolization-treated hepatocellular carcinoma after transplantation. *World J Gastroenterol* 2014; 20(37): 13538-13545 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13538.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13538

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third cause of cancer death worldwide[1]. While hepatic resection is the choice for single tumors in patients with good liver function, orthotopic liver transplantation (OLT) is considered the best first-line therapeutic approach for multiple HCCs in patients with cirrhosis, not only for the treatment of the neoplastic disease but also for resolving the organ failure. According to the most recent guidelines of the American Association for the Study of Liver Disease, the European Association for the Study of the Liver (EASL) and the European Organization for Research and Treatment of Cancer (EORTC)[2,3], OLT is recommended for patients within the so-called "Milan criteria"[4]. Some studies have shown that post-OLT survival of HCC recipients falling into the Milan criteria seems to be comparable to that of non-HCC OLT recipients[5], and thus Milan criteria have been included both in the Barcelona-Clinic Liver Cancer (BCLC) classification and in the U.S. United Network for Organ Sharing for organ procurement[6,7], although other models have been proposed (and validated), like the "up-to-seven criteria"[8].

The main limitation for OLT is the shortening of the donor pool, with the consequent need to extend the waiting list time for patients with cancer. Although large case-control studies - as well as real guidelines for HCC down-staging prior to OLT - are still lacking in the literature, some authors have emphasized the usefulness of local ablation before OLT in recipient survival[9,10], since it is likely to influence not only the drop-out rate on the waiting list, but also HCC-free survival, at least in selected cases[11-15]. Transarterial chemoembolization (TACE) is the most widely used local treatment[16-18], and it is recommended for patients with > 6 mo of expected time on the waiting list and with BCLC stage B[3,11,19]. In our institution, pre-OLT TACE is used both for down-staging in patients beyond the Milan criteria, and to delay tumor progression of patients within the Milan criteria ("bridging therapy") in order to grant more time and to reduce the drop-out rate in the waiting list.

An important issue is the objective discrepancy existing between the pre-transplant (radiological) staging and the explant staging on the OLT specimen. This discrepancy depends on the sensitivity of imaging techniques that can fail in the recognition of small-sized atypical/hypovascular HCCs[20,21]. According to Mazzaferro *et al*[8], the pre-transplant staging fails to predict the actual number of HCC in 25%-35% of patients.

Several clinical and radiological studies are present in the literature, very few analyzing the histological aspect of TACE-treated HCCs and tumor recurrence. Moreover, histological studies assessing the post-TACE tumor regression (and its meaning for HCC recurrence) are still lacking. The aims of this study were: (1) to define the histopathological features predictive of HCC recurrence in patients treated with TACE before OLT; and (2) to establish if these features can be used for defining a histological "score" predictive of HCC recurrence after OLT, that can be applied by pathologists for recipient risk stratification.

MATERIALS AND METHODS

Patient selection and clinical data

We retrospectively reviewed the specimens from consecutive OLT recipients which came to our Pathology Unit over a 6-year period (from January 2005 to December 2010). Eligibility criteria were the TACE ablation of at least one HCC before OLT and the availability of adequate follow-up data. All patients were treated according to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008); informed consent was obtained from each patient at the time of surgery. Data about HCC number and size as seen at computerised tomography (CT) and/or magnetic resonance imaging (MRI) and US analysis and the number of locally ablated nodules were collected, together with the resulting number of patients included in the Milan criteria before TACE.

One-hundred and one patients were finally selected, 85 (84.2%) males and 16 (15.8%) females, mean age at OLT 56.61 ± 7.82 years (range 37-68 years). At pre-transplant evaluation a mean number of 2.42 ± 1.19 HCC/patient was found (range 1-6), with a mean size of the largest nodule of 2.86 ± 0.98 cm (range 1.00-5.00 cm). Forty-four (43.6%) patients were classified beyond the Milan Criteria: the goal of TACE in these patients was the suc-

cessful downstaging. Conversely, 57 (56.4%) patients were within the Milan criteria, and all their HCCs were treated as "bridging therapy", to prevent drop-out from the waiting list. A total of 195 nodules were treated with TACE, for a mean of 1.93 ± 1.16 nodules/patient (range 1-6).

Follow-up data included HCC recurrence rates after OLT (both graft and systemic localizations) and recipient death rates for HCC recurrence and other causes.

Histopathological analysis

At gross examination of the explanted livers, we performed 1-cm cut sections of the whole parenchyma, and all suspicious nodules, also with a necrotic component, were described and totally sampled. After routine processing, 3-μm-thick sections were cut from all specimen blocks for routine stainings with Hematoxylin-Eosin, Reticulin, Sirius Red, and diastase-periodic acid Schiff.

For each single nodule, the following histopathological features were collected: percentage of necrosis, tumor margins (expansive/infiltrative), growth pattern (solid, acinar and/or trabecular), tumor grade according to Edmondson, peritumoral microvascular invasion, presence of clear cell or sarcomatoid areas. Nodule size was also measured on the histological section and the volume was calculated assuming a hypothetical spherical shape, with the formula $V = 4/3(\pi r^3)$ (where V is the volume expressed in cm^3 and *r* the radius of the HCC)[22-24]. Furthermore, by subtracting the necrotic percentage from the nodule volume, we obtained the viable volume of each nodule (expressed in cm^3), as previously described[25].

For each patient, the following parameters were evaluated: the total number of nodules (NNOD, considering both the necrotic and the viable nodules), the effective number of viable neoplastic nodules at OLT, the size of the major nodule, the size of the major viable nodule, the occurrence of microscopic vascular invasion (MVI), the maximum Edmondson's grade observed, the occurrence of clear cell/sarcomatous changes. We also calculated the Residual Neoplastic Volume (RNV) of each patient, defined as the sum of all viable volumes of all nodules (in cm^3), *i.e.*, the total volume of all evaluated nodules minus the necrotic portion. RNV simply represents the total volume of viable neoplasia that was present in the liver at the time of OLT.

Statistical analysis

Data are reported as means ± SD, medians, ranges and frequencies. One-way ANOVA, the Pearson's correlation, the Fisher's exact and the linear-by-linear association tests were applied. Survival data (mean survival times and rates together with their standard errors, SE) were computed by means of the Kaplan-Meier procedure and were analyzed by means of the Cox proportional hazards model. The hazard ratios (HRs) were computed together with their 95% confidence intervals (95%CIs). The multivariate linear regression and a k-means cluster analysis were also used in order to compute the standardized histological score (HS). The SPSS (Version 13.0 for Windows; SPSS Inc., Chicago, IL, USA) was used as statistical package. Two-tailed *P* values less than 0.05 were considered significant.

RESULTS

Histopathological analysis

Two-hundreds and seventy-five neoplastic or necrotic nodules were found and sampled at gross examination in the 101 patients. At histopathological analysis, 32 (31.7%) patients with 1 nodule, 27 (26.7%) with 2, 15 (14.9%) with 3, and 27 (26.7%) patients with 4 or more nodules were found (mean 2.72 ± 1.90 nodules/patient, range 1-8). The histological characteristics of the nodules examined are illustrated in Figure 1 and summarized in Table 1. Considering only the nodules with any amount of viable HCC, 33 (32.7%) patients showed 1 viable nodule, 19 (18.8%) showed 2, 8 (7.9%) showed 3, and 17 (16.8%) showed 4 or more, for a total of 190 viable nodules (mean 1.88 ± 2.00 nodules/patient, range 0-8). Most important, no viable nodules were found in 24 (23.8%) patients, among which one had MVI near necrotic nodules: thus 23 patients were completely free from cancer at the time of OLT, and none of them experienced HCC recurrence in the follow-up.

The finding of *de novo* neoplastic nodules at OLT analysis put 34 (33.7%) patients beyond the Milan criteria after TACE, probably due to the small hypovascular nodules currently undetected by dynamic MRI/CT imaging techniques[20,21], and therefore untreatable with TACE.

At histology, the mean size of the major nodule for each patient was 2.72 ± 1.29 cm (range 0.6-6.0 cm), the mean size of the major viable nodule was 2.57 ± 1.30 cm (range 0.4-5.5 cm), MVI was observed in 38 (37.6%) patients, clear cell component was present in 11 (10.9%) cases, the maximum Edmondson's grade was 1 in one case (1.0%), 2 in 26 (25.7%), 3 in 49 (48.5%) and 4 in one (1.0%) case. The mean RNV was 9.44 ± 19.14 cm^3 (range 0-106.35 cm^3).

Correlations among nodule characteristics

The histological features of the 275 nodules were correlated with each other: the presence of MVI adjacent to a nodule was significantly related to a high Edmondson's grade (76 nodules showed MVI, among which 50 had Edmondson's grade 3 or 4, $P = 0.001$, linear-by-linear association) and to infiltrative margins (in 50 nodules, $P < 0.001$, Fisher's exact test); Edmondson's grade and tumor infiltrative margins were correlated too (of 84 HCC with infiltrative margins 56, 66.7%, were grade 3 or 4, of 105 HCC with expansive margins 45, 42.8%, were grade 3 and none of grade 4, $P < 0.001$, linear-by-linear association). These findings should not be surprising, since all these features are typical of more aggressive HCCs. Nodule necrosis was directly correlated with tumor size ($P = 0.001$, Pearson's R), in line with what was previously reported in our Institution[12], and inversely related to the perinodular MVI (mean necrosis 32.8% ± 37.4%

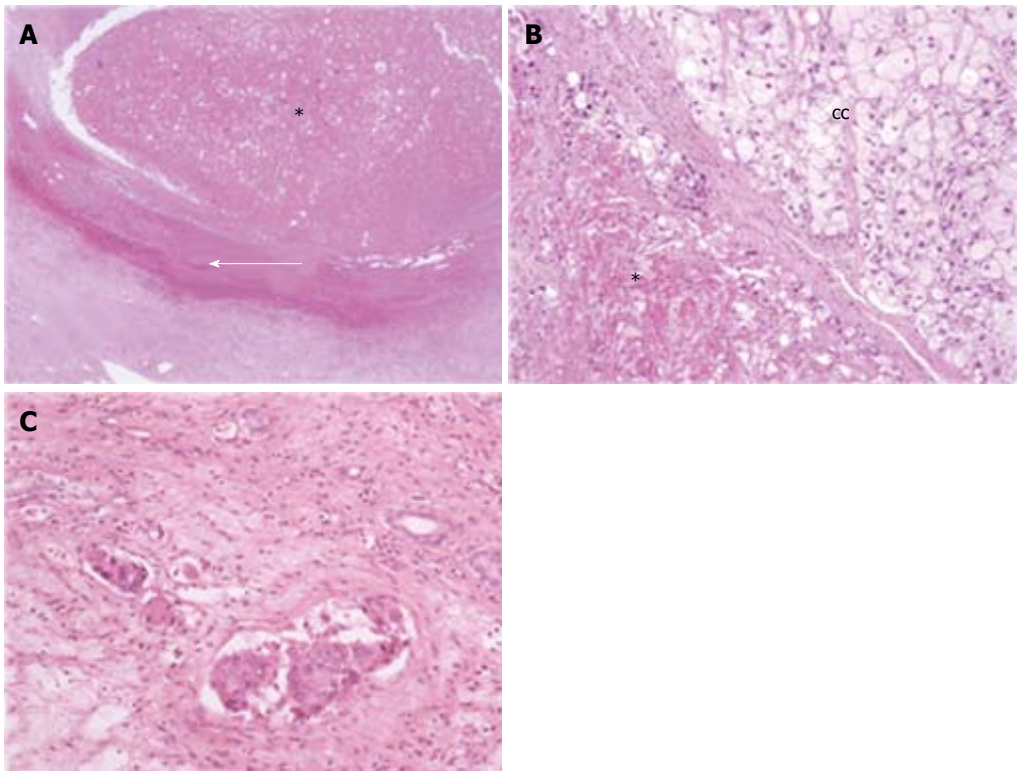

Figure 1 Different histological pictures after transarterial chemoembolization. A: A case of totally necrotic nodule (asterisk), with a well-visible fibrotic capsule (white arrow); B: A case of clear cell (CC) hepatocellular carcinoma with only partial necrosis (asterisk); C: Amicrovascular invasion. Haematoxylin-Eosin stain; Magnification × 2 (A) and × 10 (B, C).

Table 1 Histological characteristics of all examined nodules (above) and of all viable nodules (any amount of residual tumor, below) *n* (%)

Examined nodules (*n* = 275)	
Mean diameter (cm)	1.89 ± 1.18 (range 0.30-6.00)
Mean volume (cm^3)	8.59 ± 16.93 (range 0.01-113.04)
Mean viable volume (cm^3)	3.47 ± 9.56 (range 0-78.36)
Complete coagulative necrosis	85 (30.9)
Mean necrosis percentage	48.54% ± 45.58%
Microvascular invasion	81 (29.5)
Infiltrative margins	84 (30.9)
Viable nodules (*n* = 190)	
Trabecular/trabeculo-acinar pattern	129 (67.9)
Edmondson's grade:	
1	10 (5.3)
2	78 (41.1)
3	100 (52.6)
4	2 (1.1)
Clear cell changes	14 (7.4)

in the 81 nodules with thrombosis *vs* 55.1% ± 47.1% in the 194 nodules without thrombosis, $P < 0.001$, one-way ANOVA). Solid architecture was correlated with a clear cell morphology [10 of 52 (19.2%) clear-cell HCC *vs* 4 of 136 (2.9%), $P = 0.001$, Fisher's exact test].

Follow-up

Mean follow-up from OLT was 3.55 ± 1.73 years (range from 14 d to 6.81 years, median 3.37). At the end of follow-up, 14 (13.9%) recipients experienced HCC recurrence either in the graft or extrahepatic localizations, after a mean of 1.12 ± 0.89 years (range 69 d to 3.01 years, median 1.00). Twelve of these 14 (85.7%) recurrences occurred in patients discovered beyond the Milan criteria at histology. Conversely, 65 (74.7%) out of 87 not-recurrent cases occurred in patients within the Milan criteria after the histological examination.

Eighteen (17.8%) recipients died, 9 for HCC recurrence, *i.e.*, the 64.3% of the 14 recipients with HCC recurrence. The other causes of death included HCV recurrence (4 cases), sepsis/infection (3 cases), and cerebrovascular events (2 cases).

Calculation of a Histological Score (HS) predictive of HCC recurrence

At univariate Cox analysis, the only donor variables significantly related to post-OLT recurrence were: the MVI ($P = 0.002$, HR = 7.23, 95%CI: 2.01-25.94), the total number of nodules ($P < 0.001$, HR = 1.54, 95%CI: 1.25-1.89), the number of viable nodules ($P < 0.001$, HR = 1.47, 95%CI: 1.22-1.77), and the RNV ($P = 0.002$, HR = 1.03, 95%CI: 1.01-1.04). In particular, 11 out of 14 HCC recurrence cases had MVI (*i.e.*, MVI had a 78.6% sensitivity), while 60 of the 87 patients without recurrence did not show histological MVI (*i.e.*, specificity of the 69.0%). Furthermore, these variables were also confirmed as the only ones significantly predictive of HCC-related deaths after recurrence in the subset of patients who had had recurrence (data not shown). Age ($P = 0.872$), sex ($P =$

Table 2 Recurrence-free survival data and hazard ratios of hepatocellular carcinoma recurrence according to the histological score classes *n* (%)

HS risk classes	Mean recurrence-free survival time (± SE; years)	5-yr recurrence-free survival rate (± SE)	Hazard ratio (95%CI)[1]	HCC recurrence cases	HCC-related deaths
Low (HS: 0-1; *n* = 61)	6.60 ± 0.15	96.7% ± 2.3%	1.00	2 (3.3)	1 (1.6)
Moderate (HS: 1-2; *n* = 27)	5.43 ± 0.45	77.9% ± 8.9%	6.37 (1.23-32.86)	5 (18.5)	3 (11.1)
High (HS: >2; *n* = 13)	2.92 ± 0.21	40.0% ± 14.6%	23.18 (4.80-111.96)	7 (53.8)	5 (38.5)

[1]Univariate Cox proportional hazards model. SE: Standard error; CI: Confidence interval; HCC: Hepatocellular carcinoma; HS: Histological score.

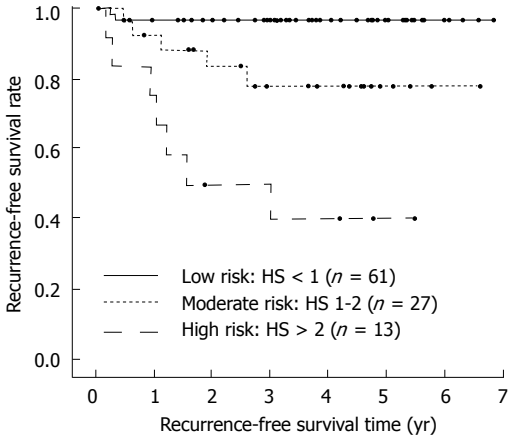

Figure 2 Recurrence-free survival according to the three histological score classes. The actuarial recurrence-free survival rate is 96.7% in the low-risk group, 77.9% in the moderate-risk group and 40.0% in group the high-risk group.

0.373), inclusion in Milan criteria prior to TACE (P = 0.639) and dimension of the major nodule (P = 0.080) were not significantly related to post-OLT recurrence.

The histological cancer variables (Edmondson's grade, clear cell component, dimension of the major viable nodule) were available only in the 77 patients with residual HCC and resulted not significantly related to post-OLT HCC recurrence (P = 0.336, P = 0.193, and P = 0.229, respectively); therefore, they have been excluded from the multivariate model. The model finally considered 101 cases and 8 variables: recipient age and sex, inclusion in Milan criteria prior to TACE, RNV, number of total nodules, number of viable nodules, MVI, dimension of the major nodule.

The number of total nodules (P = 0.073), the residual neoplastic volume (RNV, P = 0.035) and the neoplastic microvascular invasion (MVI, P = 0.062) entered the Cox multivariate hazard model with HCC recurrence as dependent variable.

For the purposes of our study, we computed a score based on predictive variables only and unrelated to time-dependent components, the histological score (HS) was computed by means of a multivariate linear regression having the rescaled hazard level evaluated by the Cox analysis in each patient as dependent variable and these three parameters as the independent ones. In order to find a solid classification of the patients into 3 groups at different risk levels, a cluster analysis was made. Finally, the coefficients of the model were standardized in order to obtain the values of 1 and 2 as cut-off separating the 3 groups at different risk levels (*i.e.*, low risk class: HS ranging from 0 to 0.99; moderate risk class: HS ranging from 1 to 1.99; high risk class: HS equal to, or greater than, 2).

The final score resulted:

$$HS = (0.164 \times NNOD) + (0.012 \times RNV) + (1.015 \times MVI)$$

where NNOD is the total number of total nodules, RNV is the residual neoplastic volume and the notation "$1.015 \times MVI$" means that the 1.015 value should be added to the sum of the previous two terms in patients with presence of microvascular invasion only.

In our population of 101 patients the HS ranged between 0.164 and 3.393 (mean 0.942 ± 0.816) showing an accuracy of 84.2% in discriminating between HCC recurrent and non-recurrent cases (AUC ± SE: 0.842 ± 0.047). By using the best cut-off (HS ranging between 0.664 and 0.667) a sensitivity of 100% (14/14) and a specificity of 60.9% (53/87) were reached. The cluster analysis sorted the population into the following three classes: 61 patients (60.4%) with low risk; 27 patients (26.7%) with moderate risk; and 13 patients (12.9%) with high risk.

The cumulative recurrence-free survival of the three classes is shown in Figure 2, while survival data, together with the hazard ratios *vs* the low risk class, are shown in Table 2. Of note, the 5-year recurrence-free survival rate decreases from the 96.7% of the low-risk group to the 77.9% of moderate-risk group and the 40.0% of high-risk group, and the HR for the moderate- and high-risk groups were 6.37 and 23.18 respectively when compared to the low-risk group.

DISCUSSION

The application of TACE in the pre-transplant setting is still controversial[25,26]. In our setting, TACE is performed according to the most recent guidelines for downstaging in patients beyond the Milan criteria, or for patients within the Milan criteria but with > 6 mo of expected time on the waiting list ("bridging therapy")[19,27]. Despite the abundance of clinical and radiological studies on the pre-transplant staging of HCC patients, studies analyzing

the histological aspect of tumor regression after TACE and its impact on HCC recurrence are still rare in the literature, since in the past few years only the percentage of TACE-induced necrosis has been histologically studied, mainly in comparison with MRI[12,28]. The principal aims of our study were to define the histopathological features predictive of HCC recurrence in patients treated with TACE, and to combine them into a histological "score" predictive of HCC recurrence after OLT.

The histological examination of the single nodules (grade, architecture, growth, *etc.*) did not provide contributions about prognosis, while the multivariate analysis of recipient variables allowed us to identify three histological parameters predictive both of HCC recurrence and HCC-related mortality: MVI, the number of nodules, and RNV. The Histological Score (HS) could be considered a variation of the well-established Milan criteria: in fact, it includes the number of nodules and the tumor volume (instead of dimensions)[2,4,6]. At any rate, the availability of the whole liver specimens allows the pathologist to sample and characterize all the suspicious nodules in each case, as well as to assess the real amount of viable HCC in each nodule, and MVI too. The first result of this approach was that the number of nodules proved to be predictive independently on their being viable or necrotic. A possible explanation is that a higher number of HCC nodules implies a higher risk of vascular or intrahepatic dissemination that can manifest itself also after the necrosis of the main nodules (and after OLT). This does not contradict what previously reported by our group, *i.e.* that partial tumor necrosis has a higher risk for HCC recurrence[29]: indeed, since the Residual Neoplastic Volume (RNV) is part of the HS too, it is evident that incomplete necrosis has a worse impact on disease-free survival than complete necrosis. At any chance, no patients with complete tumor necrosis (absence of viable tumor) experienced HCC recurrence in our series.

RNV was calculated assuming a hypothetical spherical shape of the HCCs[22-24] minus the amount of necrosis, and it is likely to be a fairly realistic model for tumor quantification. At a glance, the calculation of RNV might seem complicated, but only the nodule diameter and the percentage of necrosis, which are commonly included in every histopathological report, are needed. The presence of MVI in the HS is not surprising, since MVI is a well-known prognostic parameter of HCC recurrence both in non-transplanted patients and in OLT recipients with or without local therapy[2]. At any chance, since MVI resulted as a part of the HS, the diagnostic performance (computed as the balance between sensitivity and specificity) of MVI alone was obviously lower than HS diagnostic performance (73.8% *vs* 80.5%). In particular, the finding that no patients in the low risk HS class had MVI, suggests that the absence of MVI well predicts low risk of recurrence. Conversely, since MVI occurred similarly in the moderate risk (25/27, 92.6%) and high risk (13/13, 100%) recipients, the other two histological features (the number of nodules and the RNV) are likely to be needed to discriminate between moderate risk and high risk of HCC recurrence.

It is noteworthy that in our proposed model the inclusion in the Milan criteria prior to TACE had not an independent role in HCC recurrence prediction both at univariate and multivariate analyses: it seems that the indication to TACE (*i.e.*, down-staging *vs* "bridging therapy") did not influence tumor recurrence. This confirms previous findings from our group that the recurrence rate after downstaging to Milan criteria is similar to the recurrence rate of patients within Milan criteria[10].

The application of the Milan criteria on OLT specimens showed a diagnostic performance comparable to the HS (80.2% and 80.5%, respectively), but it should be pointed out that these values were determined by two measures of discrimination quite different between the two binary classification rules: *i.e.*, HS had higher sensitivity (100% *vs* 85.7%) while the Milan criteria had higher specificity (74.7% *vs* 60.9%). Finally, among the 34 patients who were beyond the Milan criteria, 9 (26.5%) were assigned to the low risk class (with only 1 case of HCC recurrence), 13 (38.2%) to the moderate risk class (with 4 cases of recurrence), and 12 (35.3%) to the high risk class (with 7 cases of recurrence). The recipients in our HS low risk class actually showed a low frequency of HCC recurrence comparable to the recipients within the Milan criteria (3.3% and 3.0%, respectively), while the histopathological analysis and the HS allowed once more a better stratification of the recipients with a higher risk: in fact the moderate-risk class reached a recurrence frequency assessment of 18.5% (5/27), and the high-risk class reached a recurrence frequency assessment of 53.8% (7/13), *vs* 35.3% of the Milan criteria alone. Briefly, our HS better stratifies the "moderate risk" from the "high risk" in those recipients that turn out to be beyond the Milan criteria after OLT, as for 33.7% of patients in our series. It is true that this percentage of patients with *de novo* nodules is likely to decrease in the future, with a more widespread use of hepatospecific contrast media on MRI, which have demonstrated an increased sensitivity for the detection of early atypical HCC, although we surmise that the histopathological analysis will be necessary also in the future.

In conclusion, our results strengthen the role of the pathologist in the management of HCC recipients after OLT. Despite the improvement of imaging techniques[21,25], only a thorough gross examination and histological analysis of all suspicious liver nodules can provide data about MVI and the finding of *de novo* early HCCs, which can put the patients beyond the Milan criteria. In these cases HS might prove to be useful in order to identify those patients with a higher risk of HCC recurrence. Our "score" is not meant to be a universal score, applicable to all HCC patients, but it might be useful for the pathologists in a liver transplantation setting to predict the recipient's real risk of HCC recurrence after TACE and after OLT, and to help the transplant surgeon in the post-transplant management of this group of patients.

COMMENTS

Background

Transarterial chemoembolization (TACE) of hepatocellular carcinoma (HCC) is used for down-staging before orthotopic liver transplantation (OLT) in patients beyond the Milan criteria, and to delay tumor progression in patients within the Milan criteria ("bridging therapy") in order to grant more time and to reduce the drop-out rate in the waiting list.

Research frontiers

Imaging techniques can fail in the recognition of small-sized atypical and/or hypovascular HCCs, so that a discrepancy between the radiological pre-transplant staging and the pathological post-transplant staging exists. According to the literature, the pre-transplant staging fails to predict the actual number of HCC in 25%-35% of patients. Apart from the number of neoplastic nodules, single pathological features such as microvascular invasion, tumor grade, etc. have already been described as important in the post-transplant HCC recurrence, but comprehensive histopathological studies analyzing their global prognostic impact have not been performed yet.

Innovations and breakthroughs

We retrospectively reviewed 275 suspicious nodules from 101 consecutive OLT recipients, collecting 11 histological variables. Survival data were analyzed: the total number of nodules, the residual neoplastic volume and the microvascular invasion entered the Cox proportional hazards model, with HCC recurrence as dependent variable. A standardized Histological Score (HS) was therefore computed, and a cluster analysis sorted the recipients into 3 risk groups according to the HS, with 3.3%, 18.5% and 53.8% respectively of tumor recurrence rates and 1.6%, 11.1% and 38.5% of tumor-related mortality respectively. This is the first time that histological tumor features are quantified and applied in a post-treatment HCC tumoral regression score, as it is already done in other tumors (*e.g.*, breast, colon-rectum).

Applications

A thorough gross examination and histological analysis of all suspicious liver nodules is mandatory to obtain reliable data about the real amount of tumor necrosis after TACE, the microvascular invasion, as well as to find *de novo* early HCCs. The HS might prove to be useful in order to identify those patients with a higher risk of HCC recurrence after OLT. In a liver transplantation setting, the application of a standardized tumoral regression score after TACE and after OLT, can help the transplant surgeon in the recipients management.

Peer review

In this paper the authors evaluate the prognostic impact of histopathology in post-transplant HCC recurrence after TACE treatment. The authors used appropriate methods of analysis and results are of interest.

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RETROSPECTIVE STUDY

Study of liver cirrhosis over ten consecutive years in Southern China

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Abstract

AIM: To investigate the etiology and complications of liver cirrhosis (LC) in Southern China.

METHODS: In this retrospective, cross-sectional study, we identified cases of liver cirrhosis admitted between January 2001 to December 2010 and reviewed the medical records. Patient demographics, etiologies and complications were collected, and etiological changes were illustrated by consecutive years and within two time periods (2001-2005 and 2006-2010). All results were expressed as the mean ± SD or as a percentage. The χ^2 test or Student's *t*-test was used to analyze the differences in age, gender, and etiological distribution, and one-way analysis of variance was applied to estimate the trends in etiological changes. We analyzed the relationship between the etiologies and complications using unconditioned logistic regression, and the risk of upper gastrointestinal bleeding (UGIB) and hepatocellular carcinoma (HCC) in the major etiological groups was evaluated as ORs. A *P* value less than 0.05 was considered significant. Statistical computation was performed using SPSS 17.0 software.

RESULTS: In this study, we identified 6719 (83.16%) male patients and 1361 (16.84%) female patients. The average age of all of the patients was 50.5 years at the time of diagnosis. The distribution of etiological agents was as follows: viral hepatitis, 80.62% [hepatitis B virus (HBV) 77.22%, hepatitis C virus (HCV) 2.80%, (HBV + HCV) 0.58%]; alcohol, 5.68%; mixed etiology, 4.95%; cryptogenic, 2.93%; and autoimmune hepatitis, 2.03%; whereas the other included etiologies accounted for less than 4% of the total. Infantile hepatitis syndrome LC patients were the youngest (2.5 years of age), followed by the metabolic LC group (27.2 years of age). Viral hepatitis, alcohol, and mixed etiology were more prevalent in the male group, whereas autoimmune diseases, cryptogenic cirrhosis, and metabolic diseases were more prevalent in the female group. When comparing the etiological distribution in 2001-2005 with that in 2006-2010, the proportion of viral hepatitis decreased from 84.7% to 78.3% ($P < 0.001$), and the proportion of HBV-induced LC also decreased from 81.9% to 74.6% ($P < 0.001$). The incidence of mixed etiology, cryptogenic cirrhosis, and autoimmune diseases increased by 3.1% ($P < 0.001$), 0.5% ($P = 0.158$), and 1.3% ($P < 0.001$), respectively. Alcohol-induced LC remained relatively steady over the 10-year period. The ORs of the development of UGIB between HBV and other major etiologies were as fol-

lows: HCV, 1.07; alcohol, 1.89; autoimmune, 0.90; mixed etiology, 0.83; and cryptogenic, 1.76. The ORs of the occurrence of HCC between HBV and other major etiologies were as follows: HCV, 0.54; alcohol, 0.16; autoimmune, 0.05; mixed etiology, 0.58; and cryptogenic, 0.60.

CONCLUSION: The major etiology of liver cirrhosis in Southern China is viral hepatitis. However, the proportions of viral hepatitis and HBV are gradually decreasing. Alcoholic LC patients exhibit a greater risk of experiencing UGIB, and HBV LC patients may have a greater risk of HCC.

Key words: Liver cirrhosis; Epidemiology; Etiology; Complication; Hepatocellular carcinoma; Southern China

Core tip: Liver cirrhosis (LC) is an important cause of death globally, and prevention and treatment based on etiology is fundamental. Large-sample epidemiology studies of the distribution and changes of etiology in the Southern China population are rare. This study illustrates that the major etiology of LC in Southern China is viral hepatitis, and the proportions of viral hepatitis and hepatitis B virus (HBV) are decreasing; whereas autoimmune, cryptogenic, and mixed etiology cases are increasing. Alcoholic LC patients exhibit a greater risk of suffering from upper gastrointestinal bleeding, and HBV LC patients exhibit greater risk of developing hepatocellular carcinoma.

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INTRODUCTION

Liver cirrhosis (LC) is a life-threatening worldwide health problem that is characterized by regenerated nodule development due to different liver diseases. Many patients can progress to upper gastrointestinal bleeding (UGIB), hepatic encephalopathy (HE), and hepatocellular carcinoma (HCC) in the decompensated stage. Hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, and alcohol consumption are considered to be the major global etiologies of LC[1-3].

The epidemiological distribution of the etiology of LC differs geographically and remains poorly described. Globally, approximately 57% of liver cirrhosis was due to HBV (30%) and HCV (27%) in 2006[2]. In the United States, most European countries, and Japan, HCV and alcohol are the most common causes, whereas HBV-related LC is predominant in most Asian-Pacific and African countries[2,4-8]. The epidemiology and etiology have changed dramatically because of socioeconomic development and hepatitis vaccination; however, the availability of detailed information is limited. Fleming *et al*[5] reported that the cirrhosis incidence increased from 12.05/100000 to 16.99/100000 person-years from 1992 to 2001 in the United Kingdom, and a nationwide survey of Japan reported that HCV infection accounted for 60.9% of LC in 2008, representing a decrease from 65.0% in 1998[4]. Moreover, studies have reported that different etiologies tend to result in different complications; for example, HCC occurs more often in viral hepatitis LC cases than in alcoholic and autoimmune LC cases, but the exact relationship between the LC etiology and complications has not been verified by clinical research on a large sample[2,9].

Based on this background, we designed this serial cross-sectional study to analyze the etiology, complications, and clinical features of 8080 patients with LC in our hospital and to determine an accurate etiological distribution, the proportional changes over 10 consecutive years, and the relationship between LC etiology and complications.

MATERIALS AND METHODS

Patient enrollment and data acquisition

We extracted the records of all patients admitted between January 1, 2001, and December 31, 2010, from the hospital-based electronic database of medical records. Any diagnostic or therapeutic code for cirrhosis, esophageal varices, or portal hypertension in the discharge diagnosis was included in the searching process. To minimize the potential of missing cases with underlying LC but lacking the above diagnostic codes, we performed a second search in the database with recorded non-malignant ascites, hepatic encephalopathy and hepatorenal syndrome. A final diagnosis of LC and a diagnosis of etiology were made according to the discharge diagnosis of the medical record; if no explicit etiology was mentioned, an additional etiological diagnosis was established based on the following criteria of etiology. Moreover, patients who had no clinical features of chronic liver disease but exhibited a positive pathologic diagnosis or did not undergo laboratory tests for evaluating liver function were excluded for this study. Each enrolled patient was assigned a date of diagnosis defined as the first record of liver cirrhosis over the 10-year span. For patients who had several admissions to our hospital during this period, we only collected the first record when the LC diagnosis was established. We collected clinical information for every patient, including age, gender, birth place, admission and discharge time, ID number, laboratory data, complications, and data indicating etiology diagnosis. Moreover, liver function status was evaluated by the Child-Pugh scoring system and the model for end-stage liver disease (MELD) scoring system. The ethics committees of the appropriate institutional review boards approved this study according to the Declaration of Helsinki 2000.

Table 1 Criteria for the etiological diagnosis of liver cirrhosis

Viral hepatitis
Hepatitis B virus (HBV): positive for HBsAg for at least 6 mo
Hepatitis C virus (HCV): positive for anti-HCV or HCV-RNA
HBV + HCV: HBV overlap with HCV
HBV + Hepatitis D virus (HDV): positive for HBsAg and anti-HDV
Alcoholic cirrhosis
The diagnosis was made by a history of alcohol use (⩾ 40 g/d for males and ⩾ 20 g/d for females or > 80 g/d within past 2 wk), physical examinations for signs of chronic liver disease, laboratory abnormalities [including elevation of aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase (GGT) and prothrombin time] or typical imaging features of fatty liver and cirrhosis and the exclusion of other causes of LC[10]
Autoimmune diseases
Autoimmune hepatitis (AIH)[11]
Primary biliary cirrhosis (PBC)[12]
AIH + PBC: AIH and PBC overlap syndrome
Primary sclerosing cholangitis[13]
Secondary biliary cirrhosis
Established when compatible with the diagnosis of liver cirrhosis, excluding some common causes of LC and indicated by cholestatic biomarkers (such as elevated alkaline phosphatase and GGT)
Metabolic diseases
Wilson's disease[14]
Hereditary hemochromatosis[15]
Non-alcoholic fatty liver disease[16]
Others (amyloidosis, glycogen storage disease, *etc.*)
Drugs
Established when the liver function test abnormalities related to drug intake and other causes of LC were excluded[17]
Vascular diseases
Budd-Chiari syndrome[18]
Cardiac[19]
Parasite cirrhosis
The diagnosis was based on clinical manifestations, epidemiologic features, and lab tests, excluding other types of LC; the diagnosis needed to be verified by a positive pathogenic finding
Infantile hepatitis syndrome
The diagnosis was based on criteria of Roberts[20]
Mixed etiology
HBV + Alcohol
HBV + HCV + Alcohol
Other known etiology
Cases with a definite known etiology but not listed above
Cryptogenic cirrhosis
Diagnosed only after an extensive evaluation excluded recognizable etiologies[21]

Criteria for the diagnosis of liver cirrhosis

The diagnosis of LC was made according to the discharge diagnosis of the medical record, and the diagnosis was made in accordance with the criteria below when no explicit etiology was mentioned. Patients with clinical features of esophageal varices, ascites, or hepatic encephalopathy were clinically diagnosed with LC. Moreover, LC could be confirmed by abdominal imaging (coarse parenchyma with enlargement or shrinkage of the liver; splenomegaly; ascites found by ultrasound, computed tomography, or magnetic resonance imaging) or surgical findings (laparoscopy, autopsy) combined with indicating laboratory findings (low platelet count and albumin, high level of serum bilirubin, and/or prolonged prothrombin time)[4]. The criteria for the etiological diagnosis of LC are listed in Table 1.

LC Complications

UGIB, HCC, HE, secondary infection, ascites, portal vein thrombosis, portal vein tumor thrombosis, cavernous transformation of the portal vein, hepatorenal syndrome, hepatopulmonary syndrome (HPS), and hepatic hydrothorax were included in our study as complications of liver cirrhosis. Complications were diagnosed in accordance with the complications listed in the medical record.

Statistical analysis

Data were analyzed using SPSS 17.0. All results were expressed as the mean ± SD or as a percentage. We used the χ^2 test or Student's *t*-test to analyze the differences in age, gender, and etiology distribution. One-way analysis of variance (ANOVA) was applied to estimate the changes in etiology over time. We analyzed the relationship between etiologies and complications using multivariate unconditioned logistic regression. All statistical tests were two-sided. *P*-values below 0.05 are considered significant.

RESULTS

Demographic characteristics of LC patients

Of the 8080 patients with LC in this study, 6719 (83.2%) were male, 1361 (16.8%) were female, and the ratio of males to females was 4.9:1. The average age at the time of diagnosis was 50.5 ± 13.0 years. Demographic characteristics of the LC patients are presented in Figure 1; the age distribution of LC patients is delineated as a pyramid depending on gender, and 93.5% of male patients and 89.7% of female patients were between 30-74 years old. The overall liver function stages are listed in Table 2. In our study population, 6728 (83.3%) patients were Cantonese, and 1300 (16.1%) were from other provinces. Moreover, we also collected data on 52 (0.6%) foreign patients who mostly came from southeastern Asian countries.

Etiology distribution

The etiology distribution of all LC cases is presented in Table 3. The top five LC causes were viral hepatitis (80.62%), alcohol (5.68%), mixed etiology (4.95%), cryptogenic cirrhosis (2.93%), and autoimmune diseases (2.03%). Among the remaining > 4.00% of patients, metabolic diseases and other known etiologies were identified in 1.40% and 0.73%, respectively. In addition to 106 cases of Wilson's disease, 4 cases of non-alcoholic fatty liver disease (NAFLD), and 2 cases of hereditary hemochromatosis, we also observed 1 case of amyloidosis and 1 case of glycogen storage disease. Moreover, we identified 46 (0.57%) cases caused by vascular diseases, which included Budd-Chiari syndrome (n = 31) and cardiac congestion (n = 15). In the 38 (0.47%) cases of secondary biliary liver cirrhosis, 30 involved biliary tract constructions, and the other 8 were congenital biliary atresia cases. Among the 33 (0.41%) cases of parasites, 30 were caused by *Clonorchis sinensis*, and 3 were caused by *Schistosoma japonicum*. In this study, we also identified 9 (0.11%) drug-induced LC cases, and the toxic drugs included

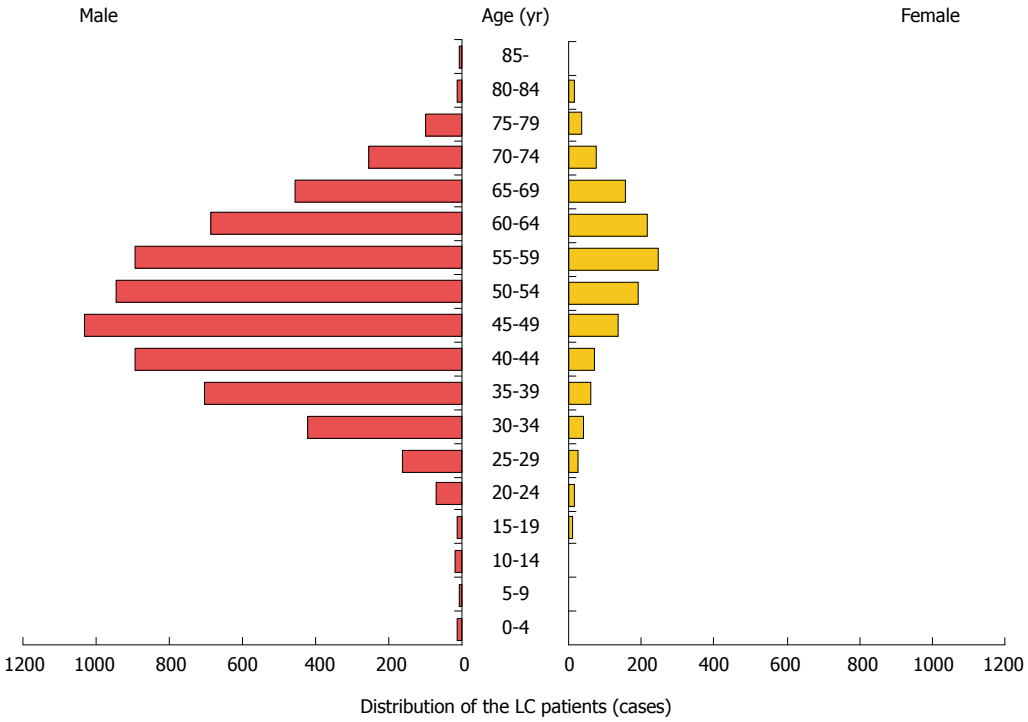

Figure 1 Demographic characteristics of the 8080 enrolled liver cirrhosis patients. Age distribution of the liver cirrhosis patients is delineated as a population pyramid depending on gender, and 93.5% of male patients and 89.7% of female patients are between 30-74 years old. Male cases are presented as red bars, and female cases are presented as yellow bars.

Table 2 Liver function characteristics of the study population *n* (%)

Male	6719 (83.2)
Mean age (yr)	50.5 ± 13.0
Child-Pugh score	
A	2521 (31.2)
B	3369 (41.7)
C	2190 (27.1)
Mean ± SD	8.0 ± 2.3
MELD score (mean ± SD)	15.3 ± 7.2
MELD: median (extreme)	13.6 (6-65)

The MELD score was calculated from 7145 cases in the study population because the creatinine value was not available from the others. MELD: Model for end-stage liver disease.

Chinese herbs, chemotherapy drugs, antipsychotic drugs, and arsenic agents. Eight cases of infantile hepatitis syndrome (IHS)-induced LC were also included in our study; the patients were neonates or infants exhibiting jaundice, light-colored feces, pathological hepatic signs, and ALT elevation with proof of liver cirrhosis.

Among the 6514 LC patients with viral hepatitis, HBV was the most common cause; HBV accounted for nearly 96% of all viral hepatitis cases and 77.2% of the study population. HCV was the second major cause of viral hepatitis LC, but the proportion was only 2.8%. In contrast, overlapping viral hepatitis involved 47 cases of HBV plus HCV and 2 cases of HBV plus HDV. The proportions were both less than 1.0% of all cases.

Etiology in different ages and genders

The etiology distribution among different ages and genders is listed in Table 3 and Table 4. With respect to the etiology in different genders, we observed that the leading etiology was viral hepatitis in both genders. However, the prevalence of viral hepatitis, alcohol, and mixed etiology was higher among men, whereas that of autoimmune disease, cryptogenic cirrhosis, and metabolic disease was higher among women ($P < 0.001$).

In all cases, IHS-induced LC patients were youngest, and the average age was 2.5 ± 3.9 years, followed by the metabolic disease group (27.2 ± 14.1 years). The parasite group had the maximum average age (58.1 ± 14.9 years), followed by the drug-induced LC group (56.0 ± 18.2 years).

Changes in the constituent ratio of etiologies from 2001 to 2010

To determine the changes in etiology from 2001 to 2010, we divided the study population into two groups. One group was defined as subjects admitted from 2001 to 2005, and the other group involved subjects admitted from 2006 to 2010. The constituent ratio of etiologies is presented in Table 5. We noticed that the top five categories did not change over the decade, but the ratio changed dramatically. The top five etiologies were viral hepatitis, alcohol, mixed etiology, cryptogenic cirrhosis, and autoimmune disease. Viral hepatitis decreased from 84.7% to 78.3% ($P < 0.001$, P-trend < 0.001); meanwhile, mixed etiology, cryptogenic cirrhosis, and autoimmune

Table 3 Etiology of the 8080 enrolled liver cirrhosis patients *n* (%)

Etiology	Total (*n* = 8080)	Male (*n* = 6719)	Female (*n* = 1361)	*P* value
Viral hepatitis	6514 (80.62)	5489 (81.69)	1025 (75.31)	< 0.001
HBV	6239 (77.22)	5316 (79.12)	923 (67.82)	
HCV	226 (2.80)	135 (2.01)	91 (6.69)	
HBV + HDV	2 (0.02)	2 (0.03)	0 (0.00)	
HBV + HCV	47 (0.58)	36 (0.54)	11 (0.81)	
Alcohol	459 (5.68)	451 (6.71)	8 (0.59)	< 0.001
Autoimmune	163 (2.03)	30 (0.43)	133 (9.77)	< 0.001
AIH	54 (0.67)	13 (0.19)	41 (3.01)	
PBC	87 (1.08)	15 (0.22)	72 (5.29)	
AIH + PBC	19 (0.24)	1 (0.01)	18 (1.32)	
PSC	3 (0.04)	1 (0.01)	2 (0.15)	
Secondary biliary	38 (0.47)	22 (0.33)	16 (1.18)	< 0.001
Metabolic diseases	114 (1.40)	73 (1.09)	41 (3.02)	< 0.001
WD	106 (1.31)	67 (1.00)	39 (2.87)	
HH	2 (0.02)	2 (0.03)	0 (0.00)	
NAFLD	4 (0.05)	2 (0.03)	2 (0.15)	
Others	2 (0.02)	2 (0.03)	0 (0.00)	
Drugs	9 (0.11)	6 (0.09)	3 (0.22)	0.186
Vascular diseases	46 (0.57)	30 (0.44)	16 (1.17)	0.001
BCS	31 (0.38)	21 (0.31)	10 (0.73)	
Cardiac	15 (0.19)	9 (0.13)	6 (0.44)	
Parasites	33 (0.41)	26 (0.39)	7 (0.51)	0.502
IHS	8 (0.10)	7 (0.10)	1 (0.07)	1.000
Mixed etiology	400 (4.95)	399 (5.93)	1 (0.07)	< 0.001
HBV + Alcohol	389 (4.81)	388 (5.77)	1 (0.07)	
HBV + HCV + Alcohol	11 (0.14)	11 (0.16)	0 (0.00)	
Other known etiology	59 (0.73)	51 (0.76)	8 (0.58)	0.499
Cryptogenic cirrhosis	237 (2.93)	135 (2.01)	102 (7.49)	< 0.001

The differences in gender proportion were determined using the χ^2 test. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; WD: Wilson's disease; HH: Hereditary hemochromatosis; NAFLD: Non-alcoholic fatty liver disease; BCS: Budd-Chiari syndrome; IHS: Infantile hepatitis syndrome.

disease increased by 3.1% (both *P* value and *P*-trend < 0.001), 0.5% (*P* value = 0.158; *P*-trend = 0.011), and 1.3% (both *P* value and *P*-trend < 0.001), respectively. The incidence of alcohol-induced LC was relatively stable over the 10-year period, although there was a slight increase from 5.3% to 5.9% in the constituent ratio (both *P* value and *P*-trend > 0.05, Figure 2). As the major cause of viral hepatitis-induced LC, the proportion of HBV-induced LC also decreased from 81.9% to 74.6% (*P* < 0.001, *P*-trend < 0.001). Compared with the decreasing proportion of HBV-induced LC, the proportion of HCV-induced LC slightly increased from 2.3% to 3.1% (*P* = 0.044; *P*-trend = 0.263, Figure 3).

LC complication distribution

Among all of the patients, we found that ascites, HCC, secondary infection, and UGIB were the most frequent complications, and the numbers of cases were 4493 (55.6%), 2399 (29.7%), 2177 (26.9%), and 1006 (12.5%), respectively. The other complications were relatively less common, and HPS was rare in our study group (6 cases). The distribution of complications classified by gender is presented in Table 6.

Table 4 Etiology of the 8080 liver cirrhosis patients by age

Etiology	Age, yr		
LC classification	mean ± SD	*t* value	*P* value
Viral hepatitis	50.7 ± 12.4		
Alcohol	52.2 ± 11.0	-1.48	0.308
Autoimmune	54.3 ± 12.9	-3.66	0.029
Secondary biliary	46.3 ± 25.1	4.33	1.000
Metabolic diseases	27.2 ± 14.1	23.49	< 0.001
Drugs	56.0 ± 18.2	-5.33	1.000
Vascular diseases	42.8 ± 17.6	7.87	0.212
Parasites	58.1 ± 14.9	-7.42	0.319
IHS	2.5 ± 3.9	48.18	< 0.001
Mixed etiology	51.2 ± 9.7	-0.52	1.000
Other known etiology	44.7 ± 13.6	5.98	0.079
Cryptogenic cirrhosis	55.0 ± 17.5	-4.29	0.015

The *P* values were determined using the independent samples *t*-test. The viral hepatitis patients were used as the reference group when comparing differences in age for different etiologies. LC: Liver cirrhosis; IHS: Infantile hepatitis syndrome.

Relationship between etiology and complications

To determine whether various etiologies might cause a variance in complications, the ORs of different etiologies for UGIB and HCC are presented in Table 7 and Table 8, respectively. We found that alcoholic LC patients exhibited a higher risk of developing UGIB compared with other major etiologies, whereas HBV infection was the most important cause of HCC in LC patients. The ORs of UGIB in all major etiologies were as follows: HBV, 1.00; HCV, 1.07; alcohol, 1.89; autoimmune, 0.90; mixed etiology, 0.83; and cryptogenic cirrhosis, 1.76. The ORs of HCC in all major etiologies were as follows: HBV, 1.00; HCV, 0.54; alcohol, 0.16; autoimmune, 0.05; mixed etiology, 0.58; and cryptogenic cirrhosis, 0.60.

DISCUSSION

In this large sample and medical center-based study, we found that viral hepatitis is the most prevalent cause of LC in our hospital, accounting for more than 80% of all LC patients. In addition, most of the viral hepatitis LC was caused by HBV (nearly 96% of all viral hepatitis LC cases). However, because of the admission of a large number of patients from other provinces of Southern China and the considerable local influence of our hospital, our single-center based findings may reflect the changing trend of etiologies in Southern China to some extent.

In the study group, the ratio of males to females is 4.9:1 (6719 males *vs* 1361 females), which is remarkably

Table 5 Etiologic analysis for the years 2001-2005 and the years 2006-2010 *n* (%)

Etiology	2001-2005 (*n* = 2907)	2006-2010 (*n* = 5173)	χ^2-value	*P* value	*P*-trend
Viral hepatitis	2462 (84.7)	4052 (78.3)	48.22	< 0.001	< 0.001
HBV	2381 (81.9)	3858 (74.6)	56.78	< 0.001	< 0.001
HCV	67 (2.3)	159 (3.1)	4.05	0.044	0.263
HBV + HCV	12 (0.4)	35 (0.7)	2.24	0.135	0.267
HBV + HDV	2 (0.1)	0 (0.0)	-	0.129	-
Alcohol	155 (5.3)	304 (5.9)	1.031	0.31	0.919
Autoimmune	34 (1.2)	129 (2.5)	16.509	< 0.001	< 0.001
Secondary biliary	12 (0.4)	26 (0.5)	0.321	0.571	0.369
Metabolic diseases	32 (1.1)	82 (1.6)	3.139	0.076	0.07
Drugs	1 (0.0)	8 (0.2)	1.459	0.227	0.161
Vascular diseases	17 (0.6)	29 (0.6)	0.019	0.89	0.551
Parasites	14 (0.5)	19 (0.4)	0.598	0.439	0.154
IHS	2 (0.1)	6 (0.1)	0.078	0.78	0.696
Mixed etiology	87 (3.0)	313 (6.1)	36.984	< 0.001	< 0.001
Other known etiology	16 (0.5)	43 (0.8)	2.95	0.086	0.285
Cryptogenic cirrhosis	75 (2.6)	162 (3.1)	1.989	0.158	0.011

The *P* values were determined using the χ^2 test by comparing the proportion of 2001-2005 with that of 2006-2010. The *P*-trend values were determined using ANOVA by comparing proportions of separated years and reflected the general trend of the etiology proportion in the 10 consecutive years.

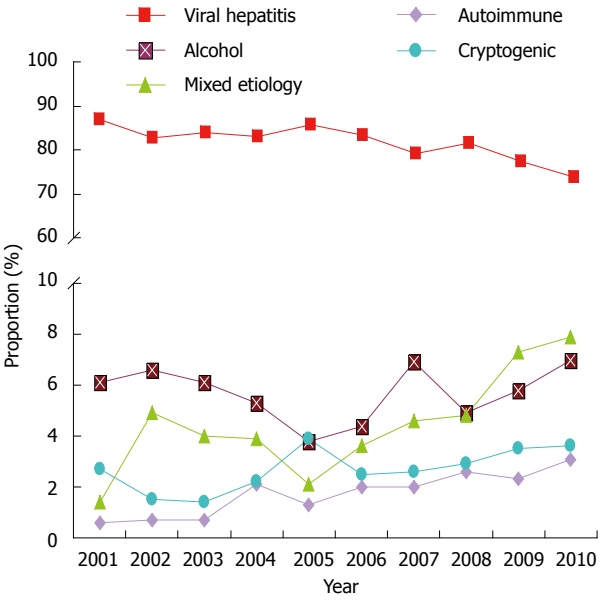

Figure 2 Changing trends in the proportion of major etiologies over 10 consecutive years. The proportion of viral hepatitis-induced liver cirrhosis (LC) has decreased gradually in the past 10 years and that of alcoholic, mixed etiology, autoimmune and cryptogenic-induced LC have increased to some extent. Every marker illustrates the proportion of LC due to a different cause, and the changing trend is simulated by lining up the markers for each etiology as different colored lines. Viral hepatitis: LC caused by viral hepatitis, $P < 0.001$ in 10 years comparison; Alcohol: LC caused by alcohol consumption, $P = 0.919$ in 10 years comparison; Mixed etiology: LC caused by mixed etiology, $P < 0.001$ in 10 years comparison; Autoimmune: LC caused by autoimmune diseases, $P < 0.001$ in 10 years comparison; Cryptogenic: LC of unknown reason, $P = 0.011$ in 10 years comparison.

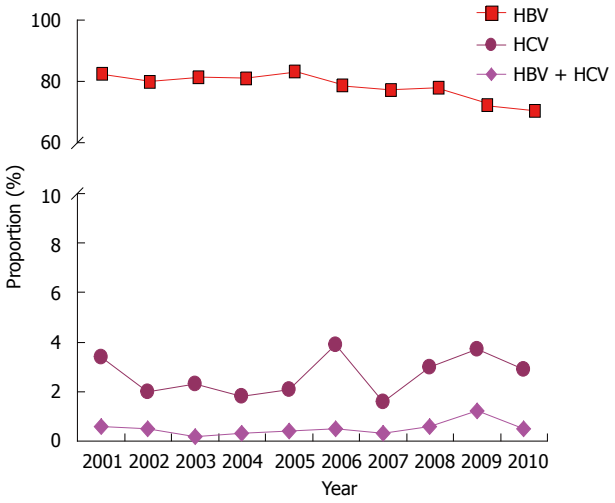

Figure 3 Changing trends in the proportion of subgroups of viral hepatitis over 10 consecutive years. The proportion of HBV-induced LC has decreased gradually over the 10 years, and those of HCV- and (HBV + HCV)-induced LC have not exhibited explicit trends. Every marker illustrates the proportion of LC due to a different cause, and the changing trend is simulated by lining up the markers for each etiology as different colored lines. HBV: LC caused by hepatitis B virus, $P < 0.001$ in 10 years comparison; HCV: LC caused by hepatitis C virus, $P = 0.263$ in 10 years comparison; HBV + HCV: LC caused by hepatitis B virus combined with hepatitis C virus, $P = 0.267$ in 10 years comparison. LC: Liver cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

higher than what is reported in some other countries[4,5]. The gender difference may be explained by the higher prevalence of HBV infection (male to female 5.8:1) in LC patients.

The HBV-predominant etiology pattern was consistent with some former studies of mainland China[22,23], Hong Kong[24], and Taiwan[25]. China is a highly endemic area of HBV infection. According to an epidemiological survey of HBV infection conducted in 2006, the hepatitis B surface antigen (HBsAg) carrier rate was 7.18% in the overall population, meaning that there was an estimated 93 million HBV carriers, 30 million of whom developed chronic hepatitis B in China[26].

In this study, we divided our research population into two groups based on admission time, before 2006 and after 2006, for a comparison of the etiology changes during the decade studied. We noticed that the ratio of viral hepatitis LC decreased slightly in the two periods (from 84.7% to 78.3%) and decreased gradually over the 10

Table 6 Complications in the 8080 liver cirrhosis patients by gender *n* (%)

Complication	Total (*n* = 8080)	Male (*n* = 6719)	Female (*n* = 1361)	χ^2 value	*P* value
UGIB	1006 (12.5)	875 (13.0)	131 (9.6)	12.00	0.001
Infection	2177 (26.9)	1806 (26.9)	371 (27.3)	0.08	0.773
Ascites	4493 (55.6)	3739 (55.6)	754 (55.4)	0.03	0.867
HCC	2399 (29.7)	2157 (32.1)	242 (17.8)	111.21	< 0.001
HE	544 (6.7)	464 (6.9)	80 (5.9)	1.90	0.168
PVT	405 (5.0)	342 (5.1)	63 (4.6)	0.51	0.477
PVTT	783 (9.7)	733 (10.9)	50 (3.7)	67.7	< 0.001
CTPV	250 (3.1)	229 (3.4)	21 (1.5)	13.13	< 0.001
HRS	293 (3.6)	252 (3.8)	41 (3.0)	1.76	0.184
HPS	6 (0.1)	4 (0.1)	2 (0.1)	1.17	0.280
HHT	398 (4.9)	337 (5.0)	61 (4.5)	0.69	0.407

Because some patients have more than one complication, number is greater than 8080; The *P* values were determined using the χ^2 test. UGIB: Upper gastrointestinal bleeding; HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; PVT: Portal vein thrombosis; PVTT: Portal vein tumor thrombosis; CTPV: Cavernous transformation of the portal vein; HRS: Hepatorenal syndrome; HPS: Hepatopulmonary syndrome; HHT: Hepatic hydrothorax.

consecutive years (as shown in Figure 2). For the HBV group, as the leading agent of viral hepatitis, we observed a similar trend (Figure 3). Our observation was confirmed by another domestic study[23]. We also observed that more patients were captured from 2006-2010 (4052) compared with 2001-2005 (2462), consistent with what was observed in the number of HBV patients (3858 in 2006-2010 and 2381 in 2001-2005). Because of the rapid development and expanding influence of our hospital, admitted patients increased each year; thus, the increase in the number of viral hepatitis cases may be due to the increase in patient enrollment rather than increased incidence, whereas the proportion was not affected by the number of hospitalized patients. The decreasing tendency should be attributed to the widespread application of HBV vaccination starting from 1996 in our nation. The HBsAg carrier rate has decreased from 10.1% in 2001 to 7.18% in 2006 in adolescents, and in children less than 5 years of age, the rate was only 0.96% overall in Chinese people and 4.91% in children from Guangdong[27,28]. Moreover, with the development of better diagnosis options and improved management of chronic liver diseases, more and more cases of rare causes of LC are being identified, which may also have played a role in reducing the ratio.

Autoimmune liver disease is composed of autoimmune hepatitis (AIH), primary biliary cirrhosis, primary sclerosing cholangitis, and overlapping syndromes. As a relatively rare disease, the proportion of autoimmune liver disease increased from 1.2% in 2001-2005 to 2.5% in 2006-2010 and increased each year in the 10-year span (Figure 2). Song *et al*[23] also found a similar increasing trend in autoimmune LC in North China. The epidemiology of AIH remains poorly characterized in China, and the exact incidence and prevalence of AIH is not known. Our research data may suggest a possible increase in the incidence and prevalence of AIH, but some large-scale nationwide epidemiology studies from Japan, Israel, Norway, and Sweden did not report a rise in the incidence and prevalence of AIH and autoimmune LC[4,29-32]. We presumed that the rapid development and wide application of diagnostic methods for detecting autoantibodies, immunoglobulin, and other hepatitis markers may be strongly correlated with the increasing rate of the disease in our study.

For LC of unknown etiology, also known as cryptogenic LC, the etiology ratio increased from 2.6% in 2001-2005 to 3.1% in 2006-2010, and the point proportion was 2.7% in 2001 and 3.6% in 2010. Since the discovery of HCV and the established diagnosis of AIH, cholestatic liver disease, and metabolic liver disease, the etiology proportion has decreased from approximately 40% to 5%[32]. Caldwell *et al*[21] found that the majority of cryptogenic cirrhosis patients were older females with Type 2 diabetes mellitus, hyperlipidemia, and obesity. Therefore, they assumed that some of these patients may have unrecognized AIH or may have progressed from nonalcoholic steatohepatitis (NASH) to LC. Similar results were observed in a Japanese study, which found that age, body mass index (BMI), serum triglyceride level, and prevalence of HCC were higher in females than in males[4]. Our data for cryptogenic cirrhosis were complicated; cryptogenic cirrhosis is more common in females (7.5% *vs* 2.0%), and the mean age is older than that for viral hepatitis (55.0 years *vs* 50.7 years) and very close to that for autoimmune diseases (54.3 years). Moreover, these cases exhibited a lower incidence of HCC compared with HBV cases (31.7% *vs* 51.4%). Because most of NASH or NAFLD cases were treated as outpatients in the clinic and few of them were admitted, we only collected 4 cases of NAFLD LC in our study group, and little is known about the prevalence of this disease in hospitalized patients. Therefore, we cannot compare cryptogenic cirrhosis with NAFLD LC. We assumed that some of these cases may bear occult AIH or viral hepatitis due to the scarcity of liver biopsy and the absence of viral hepatitis serum markers. Further studies are necessary to confirm our observations and reveal the possible mechanisms.

It is reported that UGIB is the leading cause of death for patients with alcoholic liver cirrhosis[33]. Among the major causes of LC, the OR of alcoholic LC is highest (OR = 1.89), indicating that alcoholic LC patients may have the highest risk of developing UGIB. Risk factors for UGIB include Child-Pugh classification (C > B > A), the size of varices (large > medium > small, defined as > 10 mm, 5-10 mm, and < 5 mm in diameter, respectively), and the endoscopic presence of red wale marks. In addition, alcoholic cirrhosis is one of the main factors associated with the progression from small to large varices[34]. Luca *et al*[35] have demonstrated that minor alcohol consumption will increase hepatic venous pressure gradient (HVPG) and blood flow in the collateral portal vein. Although it is controversial whether alcohol consumption increases the incidence of gastroduodenal ulcer, research by Auroux *et al*[36] suggests that recent alcohol intake favors the development of gastroduodenal erosions. Above

Table 7 Odds ratios of the different etiologies with respect to the complications of upper gastrointestinal bleeding

Complication	HBV cirrhosis (n = 6239)	HCV cirrhosis (n = 226)	Alcoholic cirrhosis (n = 459)	Autoimmune cirrhosis (n = 163)	Mixed etiology (n = 400)	Cryptogenic cirrhosis (n = 237)	Others (n = 356)
UGIB (Y/N)	739/5500	24/202	98/361	16/147	47/353	41/196	41/315
OR	1.00	1.07	1.89	0.90	0.83	1.76	1.01
95%CI		0.68-1.69	1.48-2.41	0.50-1.60	0.60-1.16	1.21-2.52	0.72-1.44
P value		0.759	< 0.001	0.713	0.282	0.002	0.938

Others: cases excluding the six previous etiologies; OR (95%CI) was calculated with a multivariate logistic model adjusted for age, gender, year, birthplace (Cantonese, Other Chinese, Foreigner), and Child-Pugh value using the stepwise forward method, and the *P* values were determined by unconditioned logistic regression. HBV: Hepatitis B virus; HCV: Hepatitis C virus; UGIB: Upper gastrointestinal bleeding.

Table 8 Odds ratios of different etiologies with respect to the complications of hepatocellular carcinoma

Complication	HBV cirrhosis (n = 6239)	HCV cirrhosis (n = 226)	Alcoholic cirrhosis (n = 459)	Autoimmune cirrhosis (n = 163)	Mixed etiology (n = 400)	Cryptogenic cirrhosis (n = 237)	Others (n = 356)
HCC (Y/N)	2118/4121	51/175	41/418	4/159	97/303	57/180	325/31
OR	1.00	0.54	0.16	0.05	0.58	0.60	0.20
95%CI		0.38-0.75	0.11-0.22	0.02-0.17	0.45-0.74	0.44-0.83	0.13-0.31
P value		< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001

Others: cases excluding the six previous etiologies. The OR (95%CI) was calculated with a multivariate logistic model adjusted for age, gender, year, birthplace (Cantonese, Other Chinese, Foreigner), and Child-Pugh value using the stepwise forward method, and the *P* values were determined by unconditioned logistic regression. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

all, the cases of alcoholic LC may have more serious varices, higher HVPG, and more gastroduodenal erosions, resulting in a higher prevalence of UGIB.

HCC is the most common form of liver cancer and has become the sixth-most common neoplasm and leading cause of death among patients with cirrhosis[37,38]. Chronic HBV infection accounts for more than 50% of all cases of HCC globally and 70% in Asia and Africa[37]. In this study, HBV LC exhibited the highest OR compared with all other major etiologies. Our observation has been confirmed by the other large-scale study conducted in the same city, in which the OR of primary liver cancer in HBV was highest for HBV, HCV, family history, aflatoxin B1, and alcohol consumption[39]. Data from other nations and regions also suggests that HBV LC is an independent risk factor for HCC development[40,41]. Although the exact mechanism of HBV infection resulting in HCC has not been well described, a large number of experiments *in vitro* and *in vivo* have demonstrated the important role of HBV infection in HCC occurrence[42,43].

In conclusion, viral hepatitis remains a major cause of LC, but the ratio has decreased to less than 80%. Meanwhile, cases of autoimmune, cryptogenic, and mixed etiology are increasing gradually, from which an increase in incidence and prevalence can be expected in the future. LC caused by alcohol consumption accounted for significantly more patients with upper gastrointestinal hemorrhage. HBV has long been considered a strong carcinogenic agent in LC patients, and our study confirmed this point of view, suggesting that more measures should be taken to prevent and manage viral hepatitis cirrhosis.

As this is a retrospective, cross-sectional study, and although we have searched cases by all means, there are still possibilities of missing cases of LC due to the lack of clinical data; furthermore, some cases had the potential of being falsely defined as "cryptogenic" because of the absence of some laboratory tests, and the epidemiologic conclusion based on a single center clinical practice is limited. Prospective, multi-center studies need to be conducted to reveal the exact incidence and prevalence of liver cirrhosis in China.

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COMMENTS

Background

Liver cirrhosis can cause gastrointestinal bleeding, liver cancer and other severe clinical complications; therefore, it is very important to prevent and treat the disease. The epidemiology and etiology have changed dynamically in the past decade, but to date, limited information about the epidemiological distribution of the etiology in China is available. It is reported that approximately 57% of liver cirrhosis is due to hepatitis B virus (HBV) (30%) and hepatitis C virus (HCV) (27%) globally, and the etiologies differ geographically. Many studies have reported that different etiologies tend to result in different complications; for example, hepatocellular carcinoma (HCC) occurs more often in viral hepatitis liver cirrhosis (LC) cases. However, the exact relationship between the LC etiology and complications has not been verified in large sample studies.

Research frontiers

The research hotspot in liver cirrhosis epidemiology is to describe the etiology distribution in different areas, to reveal the exact incidence and prevalence and to explore the prognostic factors affecting mortality.

Innovations and breakthroughs

Clinical research concerning the etiology and complications of LC in Southern China are rare and typically involve a small sample population. We investigated the etiology distribution based on a cross-sectional, 8080-patient study and

found that viral hepatitis remained the major cause of LC in Southern China. Furthermore, we observed a continuous decrease in the constitutional ratio of HBV cirrhosis as well as a slight increase of autoimmune, cryptogenic and mixed etiology over 10 years. Moreover, the authors observed that alcohol consumption is the most important risk factor for upper gastrointestinal bleeding and confirmed that HBV is a strong carcinogenic agent in LC patients using unconditioned, multivariate logistic regression incorporating age, gender and liver function status.

Applications

The study results suggest that the major etiology of LC in Southern China is viral hepatitis. However, the proportions of viral hepatitis and HBV are gradually decreasing. Alcoholic LC patients had a greater risk of experiencing upper gastrointestinal bleeding, and HBV LC patients may have more hepatocellular carcinoma.

Terminology

LC is a chronic and progressive disorder caused by varying liver diseases and characterized by regenerated nodule development. Cirrhosis can progress to upper gastrointestinal bleeding, hepatic encephalopathy, HCC and other severe clinical complications in the decompensated stage. HCC is one of the most common malignancies worldwide and is usually caused by HCV, HBV and chronic alcohol consumption. HCC is the leading cause of death among patients with cirrhosis.

Peer review

This is an interesting cross-sectional study aimed at evaluating the etiology and complications of LC in Southern China. The manuscript is generally well written and has scientific value because it discusses a relevant subject (etiology of LC) and includes a large cohort of patients.

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RETROSPECTIVE STUDY

Novel method for esophagojejunal anastomosis after laparoscopic total gastrectomy: Semi-end-to-end anastomosis

Yong-Liang Zhao, Chong-Yu Su, Teng-Fei Li, Feng Qian, Hua-Xing Luo, Pei-Wu Yu

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Abstract

AIM: To test a new safe and simple technique for circular-stapled esophagojejunostomy in laparoscopic total gastrectomy (LATG).

METHODS: We selected 26 patients with gastric cancer who underwent LATG and Roux-en-Y gastrointestinal reconstruction with semi-end-to-end esophagojejunal anastomosis.

RESULTS: LATG with semi-end-to-end esophagojejunal anastomosis was successfully performed in all 26 patients. The average operation time was 257 ± 36 min, with an average anastomosis time of 51 ± 17 min and an average intraoperative blood loss of 88 ± 46 mL. The average postoperative hospital stay was 8 ± 3 d. There were no complications and no mortality in this series.

CONCLUSION: The application of semi-end-to-end esophagojejunal anastomosis after LATG is a safe and feasible procedure, which can be easily performed and has a short operation time in terms of anastomosis.

Key words: Laparoscopic total gastrectomy; Gastrointestinal reconstruction; Semi-end-to-end esophagojejunal anastomosis; Roux-en-Y anastomosis; Gastric cancer

Core tip: Digestive tract reconstruction after laparoscopic total gastrectomy is always a challenge for surgeons. Traditional Roux-en-Y anastomosis is difficult through the small incision in laparoscopic gastrectomy and severe complications, such as anastomotic stenosis, often occur when the operation is performed improperly. The method of semi-end-to-end esophagojejunal anastomosis that we developed is easy to operate and can also prevent anastomotic stenosis and other complications effectively.

Zhao YL, Su CY, Li TF, Qian F, Luo HX, Yu PW. Novel method for esophagojejunal anastomosis after laparoscopic total gastrectomy: Semi-end-to-end anastomosis. *World J Gastroenterol* 2014; 20(37): 13556-13562 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13556.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13556

INTRODUCTION

Compared with conventional open radical gastrectomy (OG), laparoscopic radical gastrectomy (LG) is a minimally invasive surgical approach with the significant advantages of a short recovery time and fewer postop-

Table 1 Characteristics and operative data of patients undergoing laparoscopic total gastrectomy

Characteristic	Data
Gender (male/female)	15/11
Age (yr)	58.6 ± 13.4
Body mass index (kg/m^2)	23.5 ± 5.1
Operation time (min)	257 ± 36
Anastomotic time (min)	51 ± 17
Blood loss (mL)	88 ± 46
Postoperative hospital stay (d)	8 ± 3
Complication	0

Gastrectomy reconstructed with semi-end-to-end anastomosis.

erative complications. Therefore, LG has been gradually adopted worldwide and has achieved a relatively good clinical efficacy[1-4]. With improved operative experience of the surgeons, the laparoscopy-assisted gastric radical total gastrectomy has also been more commonly performed in clinical practice[5-7]. Esophagojejunal Roux-en-Y anastomosis can leave patients with a relatively good nutritional status and ideal body weight after surgery; therefore, it has been widely used in gastrointestinal reconstruction after total gastrectomy[8,9], consisting of end-to-side esophagojejunal anastomosis combined with side-to-side jejunojejunal anastomosis. However, it is difficult to perform laparoscopic anastomosis, especially if the end-to-side esophagojejunal anastomosis is at a high level, which requires a longer operating time. In addition, severe complications, such as anastomotic stenosis, can occur if the operation is improperly performed[10]. Therefore, we developed a simple and safe method of laparoscopic semi-end-to-end esophagojejunal anastomosis that can be performed easily and requires a short operative time for anastomosis. This method can also prevent anastomotic stenosis and other complications effectively.

MATERIALS AND METHODS

Patients

This group included 26 patients with gastric cancer. The characteristics of the patients are shown in Table 1. All patients underwent preoperative routine examinations, including upper gastrointestinal tract X-ray (barium-meal), electronic gastroscopy or endoscopic ultrasonography, to identify the tumor location and pathological type. The diagnosis of gastric cancer was confirmed by pathological examination. The tumor was located at the gastric cardia in seven cases, at the bottom of the stomach in 10 cases, and in the body of the stomach in 9 cases. No hepatic, pulmonary, or other distant metastases were found in the preoperative examinations.

Surgical technique

The patients were placed in the supine position with their legs spread. Five trocars were used routinely. The surgeon stood on the left side of the patient, the surgeon assistant stood on the right side, and the laparoscope-holder assistant stood between the patient's legs. After a pneumoperitoneum was established and a lymphadenectomy was performed by laparoscopy, the esophagus was isolated for at least 5 cm over the upper edge of the tumor mass to ensure the edge of the dissection was tumor tissue-free. After a complete isolation, the duodenum was dissected using an intracorporeal linear cutter stapler[2].

Before gastrointestinal reconstruction, a small incision of 5-7 cm was made at the subxiphoid. A 21- or 25-mm circular stapler (DST EEA, Covidien llc, Mansfield, MA, United States) was selected, and a thread was passed through the small hole at the center rod tip of the anvil as the retraction thread. The anastomosis anvil, with the thread attached, was inserted into the abdominal cavity for future use. After the incision, the wound was protected by an incision closure device, and the pneumoperitoneum was re-established. Under laparoscopy, the stomach was pulled down. The longitudinal incision of the esophagus was made 3 cm above the tumor with an ultrasonic harmonic scalpel, and the anvil with the thread was completely inserted into the proximal end of the esophagus. The retraction thread was then lifted close to the esophagus incision edge, and a linear cutter stapler was used to dissect the esophagus. The center rod of the anvil was then pulled out to complete the anvil placement (Figure 1A-C and Figure 2A-C).

The jejunum was extracted through a small incision of the abdominal wall. A small mesenteric incision was made at approximately 20 cm to the ligament of Treitz. Without the isolation of the small intestinal mesentery, the jejunum was dissected with the linear stapler. A 3-cm longitudinal incision was made at the contralateral side of the mesentery, which was approximately 10-15 cm from the distal jejunum end. The circular stapler was then inserted through this incision, and the center puncture rod of the circular stapler was passed through the contralateral side of the jejunal mesentery (Figure 1D and E; Figure 2D and E). The circular stapler was placed into the abdominal cavity. After the abdominal cavity was closed with the incision closure device, the pneumoperitoneum was re-established. The center cone of the anvil and the circular stapler were connected and rotated to approach each other to complete the semi-end-to-end esophagojejunal anastomosis under laparoscopic view (Figure 1F and G; Figure 2F and G). The jejunum was again brought out of the abdominal cavity, and the longitudinal incision of the distal jejunum was closed with a linear cutter stapler transversely (Figure 2H). Then, a similar approach was used to perform side-to-side anastomosis of the proximal and distal end of the jejunum. The linear cutter stapler was used 40-50 cm adjacent to the esophagojejunal anastomosis site to complete the Roux-en-Y anastomosis (Figure 1H).

RESULTS

Twenty-six patients successfully underwent laparoscopic total gastrectomy (LATG) without the need for conversion to open abdominal surgery during the operation

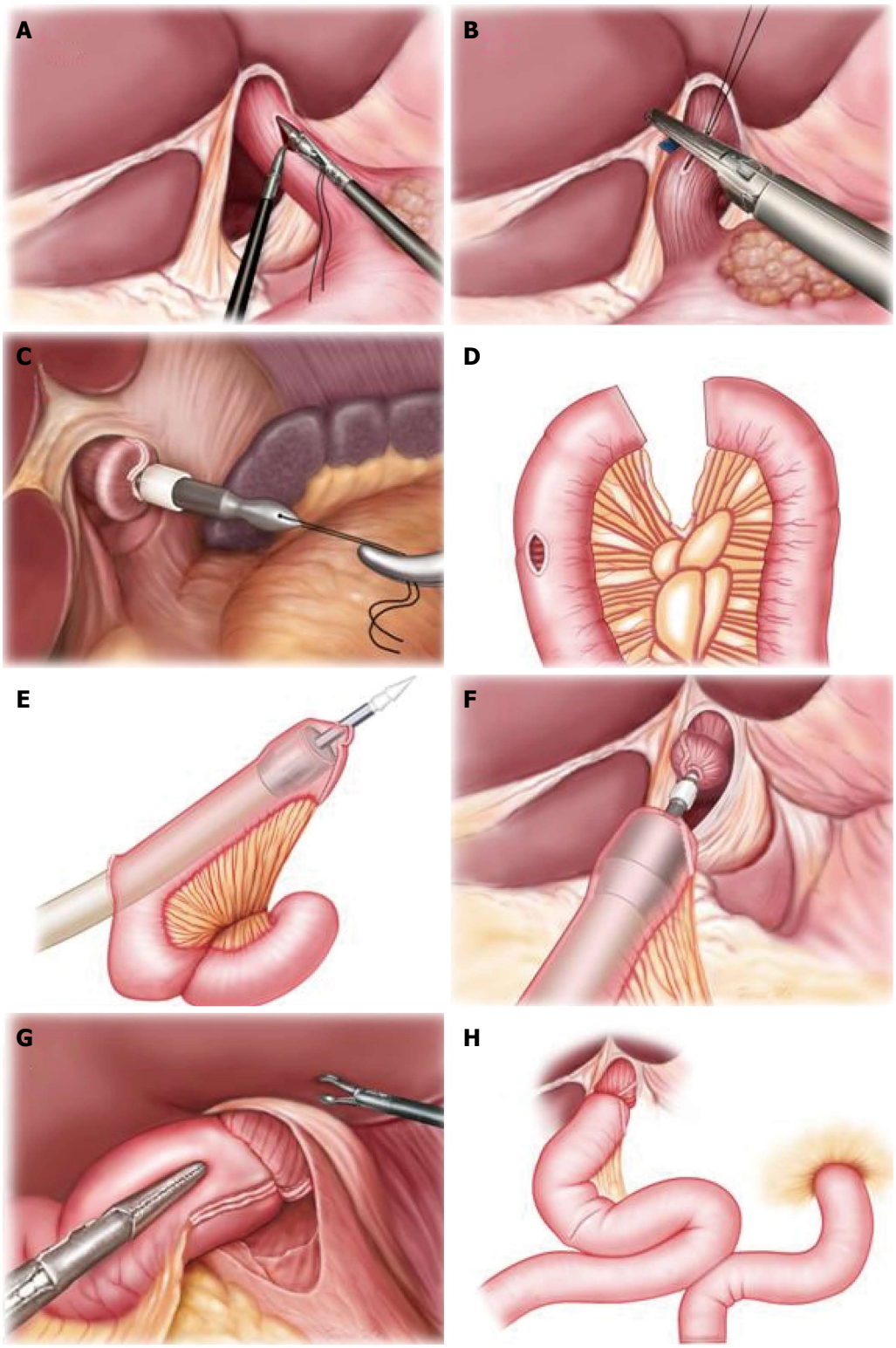

Figure 1 Diagram of gastrointestinal reconstruction after laparoscopic total gastrectomy. A: The anvil is inserted into the esophagus; B: The esophagus is dissected; C: The anvil is pulled out from the esophagus; D: The jejunum is transected, and a 3-cm longitudinal incision is made at 10-15 cm from the jejunum distal end; E: The circular stapler is inserted into the jejunum; F: Esophagojejunal anastomosis is performed; G: Semi-end-to-end esophagojejunal anastomosis is completed; H: Roux-en-Y anastomosis is completed.

(Table 1). The semi-end-to-end esophagojejunal anastomosis was also successfully performed. No residual tumor was detected at the margin of the anastomosis. The average operation time was 257 ± 36 min (range, 217-356 min), with an average anastomosis time of 51 ± 17 min and an average intraoperative blood loss of 88 ± 46 mL. A liquid diet was administered on postoperative d3, and a semi-liquid diet was administered on postoperative d5.

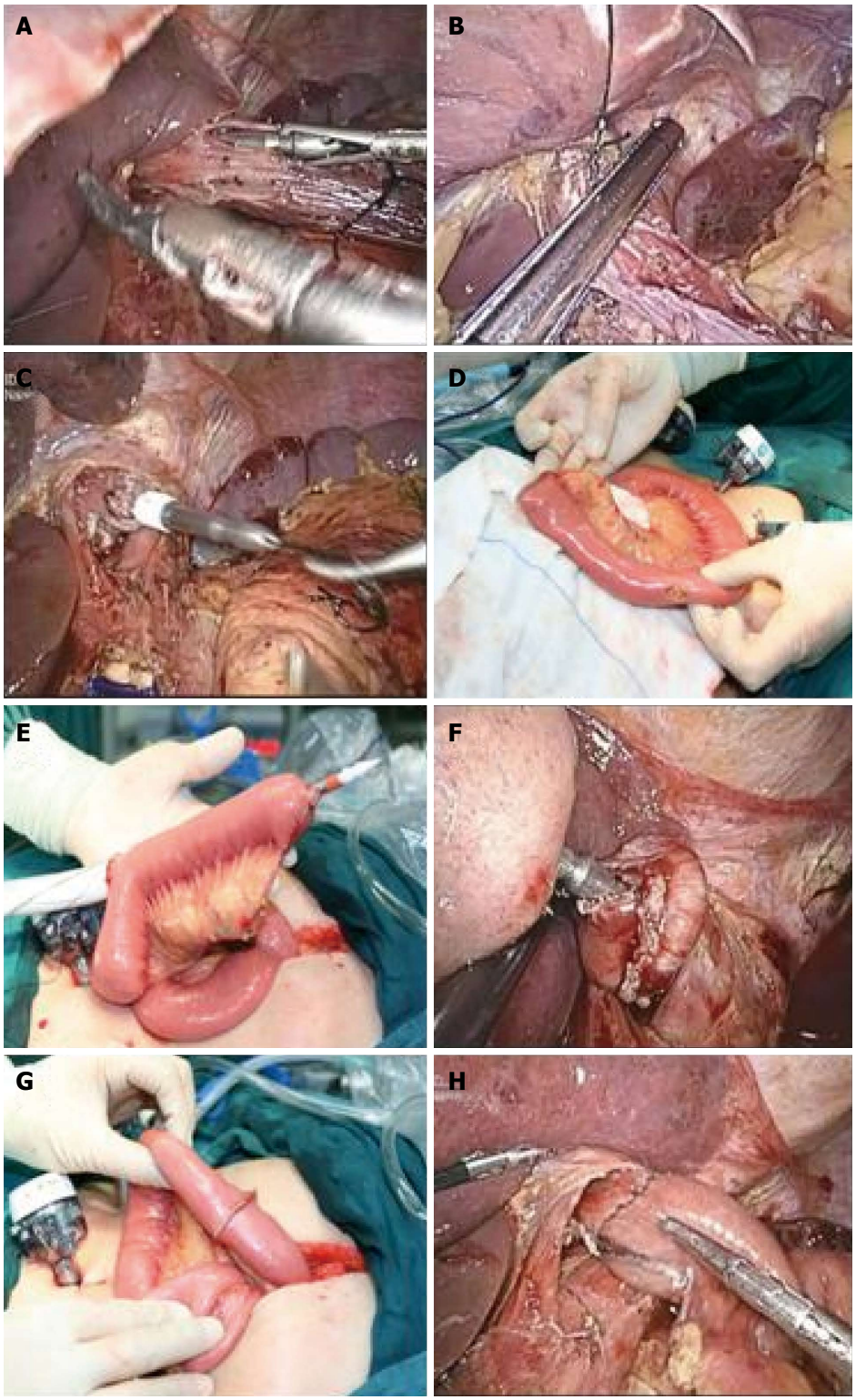

Figure 2 Surgical diagram of esophagojejunal anastomosis after laparoscopic total gastrectomy. A: The anvil is inserted into the esophagus; B: The esophagus is dissected; C: The anvil is pulled out from the esophagus; D: The jejunum is transected, and a 3-cm longitudinal incision is made at 10-15 cm from the jejunum distal end; E: The circular stapler is inserted into the distal jejunum; F: Esophagojejunal anastomosis is performed; G: Semi-end-to-end esophagojejunal anastomosis is completed; H: The jejunum incision is closed transversely.

The average hospital stay was 8 ± 3 d after surgery. No postoperative complications, such as anastomotic bleeding or leakage, were found. Barium meal examination performed after the surgery demonstrated normal perfusion, without anastomosis-site stenosis (Figure 3).

DISCUSSION

Esophagojejunal Roux-en-Y anastomosis is generally performed for digestive tract reconstruction after total gastrectomy, and the circular stapler is routinely used for

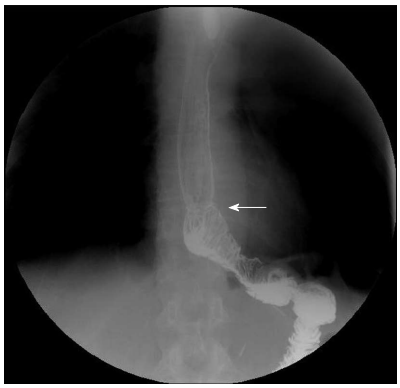

Figure 3 Postoperative Barium meal examination demonstrates normal perfusion after semi-end-to-end esophagojejunal anastomosis. An open arrow indicates the anastomosis site.

end-to-side esophagojejunal anastomosis[11-14]. In conventional open abdominal surgery, it is relatively easy to complete esophagojejunal anastomosis because of the large incision and hand-guided assistance. However, it is much more difficult to perform this procedure through a small incision with the assistance of laparoscopy. Therefore, laparoscopic esophagojejunal anastomosis after LATG has become a challenge for surgeons in laparoscopic surgery[15-18].

There are generally two technical difficulties in esophagojejunal anastomosis after LATG: anvil insertion, which has been successfully resolved using various placement methods[8,19-22], and esophagojejunal anastomosis. One method for end-to-side esophagojejunal anastomosis has been commonly applied in the literature, although this approach has several disadvantages. First, if end-to-side esophagojejunal anastomosis is performed through an incision window, the assisting incision should be selected below the subxiphoid, which requires an incision with a sufficient length because it is difficult to safely complete anastomosis through a small incision[23]. Second, because the circular stapler is inserted from the distal end of jejunum to perform end-to-side esophagojejunal anastomosis, the wall of the distal jejunum could potentially be stapled during anastomosis if the operation is not performed properly, which could cause subsequent anastomosis-site stenosis[24,25]. The above incident is more likely to occur when the anastomosis procedure is performed through a small incision or under laparoscopy because of occlusion of the liver and intestine. In addition, the operation time for this procedure is relatively long. Third, when the esophagojejunal anastomosis must be performed at a higher position, the anastomotic site will be above the diaphragmatic hiatus. In that case, there is a greater risk of anastomosis leaks from high anastomosis tension when the jejunum and mesentery near the anastomotic site can block the esophagus hiatus, resulting in postoperative symptoms such as obstruction-like sensations during eating and other type of discomfort. Furthermore, all of the above-mentioned disadvantages are more likely to present in obese patients, patients with narrow costal arch, and patients receiving esophageal dissection at a high plane.

For these reasons, we developed a method for semi-end-to-end anastomosis. The key concept in this surgical operation is that the circular stapler is inserted through a small incision of the jejunum, which is located 10-15 cm from the distal end on the contralateral side of the mesentery. In addition, the center puncture rod of the circular stapler must pass through to the jejunal end, which is also on the contralateral side of the mesentery. In such an anastomosis procedure, the anastomosis at the jejunum side is connected between the sidewall of the jejunum and the jejunal end. We therefore termed this procedure semi-end-to-end anastomosis. The anastomosis at the esophagojejunal site is at some distance from the jejunal mesentery, which can reduce the risk of anastomotic bleeding. We have also attempted to perform complete end-to-end esophagojejunal anastomosis, although if the isolated jejunal end is relatively long, a complete end-to-end anastomosis can lead to poor anastomotic vascular circulation, thereby increasing the risk of anastomotic leaks. If the mesentery is not isolated, anastomotic bleeding can easily occur after the anastomosis is completed. In our operation procedure, the jejunal mesentery is not isolated when the jejunum is dissected; however, the anastomotic site remains a certain distance from the mesentery, and it therefore does not cause anastomotic bleeding.

There are three main advantages to this semi-end-to-end esophagojejunal anastomosis. First, the procedure is simple, and the anastomosis time is significantly reduced. The entire procedure only requires a small incision, and the semi-end-to-end esophagojejunal anastomosis can be performed either through a small incision opening or under laparoscopy. As long as the stapler is connected to the center rod of the anvil, the anastomosis can be completed through rotation and tightening, which does not require repeated adjusting of the intestinal or mesenterial location under the operation window, and has no risk of anastomosis-site stenosis. The small incision in the distal jejunum can be brought out of the abdominal cavity for easy closure. Compared with the conventional end-to-side anastomosis operation, the semi-end-to-end esophagojejunal anastomosis is easy to perform and significantly reduces the anastomosis time, and the whole procedure can be successfully completed through a small incision. Second, the semi-end-to-end esophagojejunal anastomosis can effectively reduce the risk of anastomosis-site stenosis. In conventional esophagojejunal anastomosis, a stapler is required for insertion through the jejunal end, and the size of the anastomosis-site does not reflect the size of the channel for food passage after esophagojejunal anastomosis. As a result, anastomotic stenosis frequently occurs. However, by performing semi-end-to-end esophagojejunal anastomosis, the size of the anastomosis-site is equivalent to the size of the channel for food passage, which is also the actual size of the stapler. Therefore, anastomotic stenosis will not occur as long as the anastomosis is completed successfully. Third,

semi-end-to-end esophagojejunal anastomosis reduces the anastomotic tension, which reduces the risk of anastomotic leaks. The anastomosis site can also be easily retracted to the esophagus hiatus after the esophagojejunal anastomosis. Therefore, this method of semi-end-to-end esophagojejunal anastomosis can effectively reduce the anastomotic tension in obese patients and patients receiving esophageal dissection at a high plane, thereby reducing the risk of anastomotic leaks. However, our study also has some limitations. This study enrolled relatively few patients and it lacks a proper control group to assess the clinical efficacy of this new method. Despite these, we still believe this is an important basis for subsequent studies and large-scaled, prospective, randomized, controlled trials are needed to evaluate the short-term and long-term outcome of this new method.

In conclusion, the application of semi-end-to-end esophagojejunal anastomosis after LATG is a safe and feasible procedure, which can be easily performed and has a short operation time for anastomosis. In addition, this procedure can effectively reduce the incidence of anastomotic stenosis and other postoperative complications.

COMMENTS

Background

Digestive tract reconstruction after laparoscopic total gastrectomy (LATG) is a problem for surgeons. The traditional reconstruction method is difficult and severe complications, such as anastomotic stenosis, often occur if the operation is performed improperly.

Research frontiers

Digestive tract reconstruction after total gastrectomy is a clinical research focus. There are a few different approaches for esophagojejunal anastomosis, such as end-to-side esophagojejunal anastomosis, side-to-side esophagojejunal anastomosis and semi-end-to-end esophagojejunal anastomosis.

Innovations and breakthroughs

Conventional esophagojejunal Roux-en-Y anastomosis in open abdominal surgery is relatively difficult to perform through a small incision with the assistance of laparoscopy and often results in surgical complications. However, the method that we developed is not only easy to perform through a small incision, but can also effectively avoid anastomotic stenosis and reduce the risk of anastomotic leaks.

Applications

This study may represent a future strategy for digestive tract reconstruction after LATG in gastric cancer.

Peer review

The number of the patients is relatively small, and would be better with a larger number of participants. The discussion part is good.

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CLINICAL TRIALS STUDY

Factorial study of moxibustion in treatment of diarrhea-predominant irritable bowel syndrome

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Abstract

AIM: To identify an appropriate therapeutic regimen for using aconite cake-separated moxibustion to treat diarrhea-predominant irritable bowel syndrome (D-IBS).

METHODS: A factorial design was employed to examine the two factors of moxibustion frequency and number of cones. The two tested frequencies were three or six moxibustion sessions per week, and the two tested doses were one or two cones per treatment. A total of 166 D-IBS patients were randomly divided into four treatment groups, which included each combination of the examined frequencies and doses. The bilateral Tianshu acupoints (ST25) and the Qihai acupoint (RN6) were selected for aconite cake-separated moxibustion. Each patient received two courses of treatment, and each course had a duration of 2 wk. For each group, the scores on the Birmingham irritable bowel syndrome (IBS) symptom questionnaire, the IBS Quality of Life scale, the Self-Rating Depression Scale (SDS), the Self-Rating Anxiety Scale (SAS), the Hamilton Depression (HAMD) scale, and the Hamilton Anxiety (HAMA) scale were determined before treatment, after the first course of treatment, and after the second course of treatment.

RESULTS: The symptom, quality of life, SDS, SAS, HAMD, and HAMA scores of the patients in all 4 aconite cake-separated moxibustion groups were significantly lower after the first and second courses of treatment than before treatment ($P < 0.001$ for all). The symptom, quality of life, SDS, SAS, HAMD, and HAMA scores of the patients in all four aconite cake-separated moxibustion groups were significantly lower after the second course of treatment than after the first course of treatment ($P < 0.001$ for all). Between-group comparisons after the second course of treatment revealed that the symptom scores for group 1 (1 cone, 3 treatments/wk) and group 3 (2 cones, 3 treatments/wk) were significantly lower than that for group 2 (1 cone, 6 treatments/wk) (5.55 ± 5.05 *vs* 10.45 ± 6.61, $P < 0.001$; 5.65 ± 4.00 *vs* 10.45 ± 6.61, $P < 0.001$). Regarding the two levels of the two examined factors for aconite cake-separated moxibustion, after the first course of treatment, the changes in HAMA scores were

significantly different for the two tested moxibustion frequencies (P = 0.011), with greater changes for the "6 treatments/wk" groups than for the "3 treatments/wk" groups; in addition, there were interaction effects between the number of cones and moxibustion frequency (P = 0.028). After the second course of treatment, changes in symptom scores for the 2 tested moxibustion frequencies were significantly different (P = 0.002), with greater changes for the "3 treatments/wk" groups than for the "6 treatments/wk" groups.

CONCLUSION: An aconite cake-separated moxibustion treatment regimen of 3 treatments/wk and 1 cone/treatment appears to produce better therapeutic effects for D-IBS compared with the other tested regimens.

Key words: Diarrhea-predominant irritable bowel syndrome; Aconite cake-separated moxibustion; Factorial design; Moxibustion quantity; Clinical research

Core tip: This is a clinical factorial study focusing on aconite cake-separated moxibustion in the treatment of diarrhea-predominant irritable bowel syndrome. What is the effect of this ancient therapy on diarrhea-predominant irritable bowel syndrome patients? Which is the key factor in the treatment, the number of cones or the moxibustion frequency? This paper details answers to these questions.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. Epidemiological surveys have indicated that the worldwide incidence of IBS is approximately 10%-15%[1], the incidence of IBS among East Asians is approximately 5%-10%[2], and the incidence of IBS is higher among females than among males[3]. With respect to age distribution, IBS is mainly found among young adults[4]. In addition, this disease is more common among brain workers than among manual workers[5]. IBS causes tremendous mental stresses and heavy financial burdens for patients. Therefore, it is necessary to search for effective and rational therapeutic regimens for IBS.

In recent years, complementary and alternative medicine (CAM) has become widely accepted. CAM-based approaches have exhibited extremely good efficacy for treating chronic diseases. In particular, a study has revealed that 51% of IBS patients have received CAM-based treatment[6]. Moxibustion is an important treatment approach in CAM that has been widely applied in clinical contexts to treat various health issues, including gastrointestinal problems, cancer pain, insomnia and other diseases[7-9]. We have been committed to the study on technology and clinical application of treating bowel diseases with moxibustion for a long time, and have achieved innovative achievements[10]. Previous studies have indicated that moxibustion can significantly improve abdominal pain, diarrhea, and other symptoms in IBS patients[11,12] and in a rat model of IBS[13-17].

Herb cake-separated moxibustion is an indirect moxibustion therapy with a variety of materials[18]. Previous studies have demonstrated that herb cake-separated moxibustion can improve the pain thresholds of rats with chronic visceral hypersensitivity and reduce 5-hydroxytryptamine (5-HT) expression in the colonic mucosa of rats, thereby relieving the symptoms of abdominal pain and diarrhea[19]. Clinical studies have confirmed the efficacy of herbal cake-separated moxibustion in IBS patients[20,21]; however, the relationship between the quantity of stimulation with herb cake-separated moxibustion and the resulting therapeutic efficacy has not yet been established.

The number of cones and the moxibustion frequency are important factors that affect the quantity of moxibustion stimulation a patient receives. In this study, a factorial design was used to examine these two factors. In particular, the Birmingham IBS symptom questionnaire, the IBS-Quality of Life Scale (IBS-QOL Scale), the Self-Rating Depression Scale (SDS), the Self-Rating Anxiety Scale (SAS), the Hamilton Depression Scale (HAMD Scale), and the Hamilton Anxiety Scale (HAMA Scale) were used to evaluate the clinical efficacy of aconite cake-separated moxibustion treatments involving different moxibustion doses and frequencies in IBS patients. This approach allowed the selection of the optimal examined aconite cake-separated moxibustion regimen for IBS treatment, thereby providing a reliable basis for treating IBS with aconite cake-separated moxibustion in clinical practice.

MATERIALS AND METHODS

Subjects

All patients were treated at the medical outpatient department of the Shanghai Research Institute of Acupuncture and Meridian or at community health service stations in Fenglin, which is located in the Xuhui District of Shanghai, between July 2010 and April 2012. This clinical trial was registered in the Chinese Clinical Trial Register (registration number: ChiCTR-TRC-10000887) and was reviewed and approved by the ethics committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, which is affiliated to Shanghai University of Traditional Chinese Medicine (approval number: 2010-01).

The study subjects were patients between 18 and 65 years of age who were diagnosed with diarrhea-predominant IBS (D-IBS) based on the Rome Ⅲ diagnostic criteria[22], who volunteered to participate in

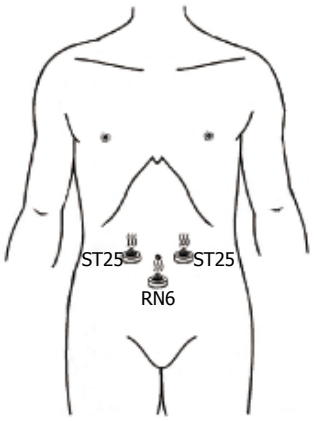

Figure 1 Diagram of aconite cake-separated moxibustion. ST25: Tianshu acupoint; RN6: Qihai acupoint.

this trial and signed informed consent forms. Patients with organic diseases of the intestinal tract and patients whose conditions were complicated with heart, liver, or kidney disease and/or mental illness were excluded from the study. Patients with constipation-predominant IBS or mixed IBS were excluded from this investigation. Patients who were currently being treated with Dioctahedral Smectite, Pinaverium Bromide Tablets, Cisapride or traditional Chinese medicine were also excluded.

Methods

A factorial design approach was used to examine the two factors of moxibustion frequency and number of cones. There were 41 patients in aconite cake-separated moxibustion group 1 (1 cone of moxibustion per treatment, 3 treatments/wk), 42 patients in group 2 (1 cone of moxibustion per treatment, 6 treatments/wk), 42 patients in group 3 (2 cones of moxibustion per treatment, 3 treatments/wk), and 41 patients in group 4 (2 cones of moxibustion per treatment, 6 treatments/wk).

Treatment method

The bilateral Tianshu acupoints (ST25) and the Qihai acupoint (RN6) were selected for the moxibustion treatments in this study. The positioning of the acupoints was based on the national standards of the People's Republic of China, which are set forth in "Nomenclature and location of acupuncture points" (GB/T12346-2006). According to traditional Chinese medicine theory, the Tianshu and Qihai acupoints can regulate gastrointestinal functions; moreover, our previous studies have confirmed that these acupoints can be used to effectively treat IBS[19].

The aconite cakes used in the aconite cake-separated moxibustion groups were produced as follows. Aconite (Radix Aconitilateralis Preparata, Sichuan) was ground into powder, sieved with a 100-mesh sieve, and yellow rice wine was added to make a thick paste (with a 5:6 ratio of aconite powder to yellow wine). The paste was pressed into a mold to form aconite cakes with a diameter of 28 mm and a thickness of 5 mm. Refined pure moxa sticks were selected (Hanyi, Nanyang, China; size: 17 mm × 200 mm) and cut into moxa cones with a height of 16 mm and a weight of 1.8 g; these cones were then used to perform aconite cake-separated moxibustion.

The moxa cones were placed on top of the aconite cakes and ignited. Moxibustion was then performed by placing the aconite cakes atop the Tianshu (ST25) and Qihai (RN6) acupoints on the patient's abdomen (Figure 1). One cone referred to the dosage received when a moxa cone had completed burning naturally. Moxibustion was performed on the patients in each group in accordance with the aforementioned frequencies and numbers of cone. Each course of treatment lasted 2 wk, and the patients received two courses of treatment.

Questionnaires

Patients were asked to complete the following questionnaires:

IBS symptom severity scale: This scale was initially developed and validated by Francis *et al*[23]. The scoring system is primarily used to assess patients' abdominal pain severity and frequency, abdominal distention severity, defecation feelings, and quality of life. We used a Visual Analogue Scale (VAS) to score the aforementioned five questions, with higher scores indicating more severe symptoms. A previous study classified severity scores for IBS as follows: scores lower than 175 indicate mild IBS symptoms, scores between 175 and 300 indicate moderate, and scores higher than 300 indicate severe[23].

Birmingham IBS symptom questionnaire: This scale[24], which is based on symptoms that frequently occur in IBS cases, examines abdominal discomfort status, stool properties, and defecation feelings. The scale includes a total of 14 items, with 6 possible grades for each item that range from 0 points (never having this symptom) to 5 points (the symptom is always present). The total score is the sum of the scores for each item.

IBS-QOL scale: The IBS-QOL scale was formulated and developed by Drossman *et al*[25] to assess the extent to which IBS symptoms have affected the following nine aspects of a subject's quality of life within the prior month: emotional state, psychological state, sleep, energy, daily activities, eating habits, social activities, major activities or work, and sex life. The scale contains 34 items, with each item measured independently based on the patient's subjective feelings. A 5-point scale ranging from 1 point (the symptom has never occurred) to 5 points (the symptom's description is completely accurate) was used for each item. Each item was scored separately, and then, the points for all items were summed to obtain a total score. Lower scores indicate better quality of life for patients, whereas higher scores indicate worse quality of life[26].

SDS: The Zung SDS is the prototype of the SDS[27]. The

advantages of the SDS include its ease of use and ability to directly reflect patients' subjective feelings. In particular, the SDS can assess specific feelings associated with the depressive states of patients in the four experimental groups; the phenomena detected by this scale include spiritual emotions, somatic disorders, psychomotor disorders, and psychological disorders. The SDS contains a total of 20 items that measure a subject's depression level during the prior wk. Each item was scored on a scale ranging from 0 = never to 4 = very often, and the total raw score was the sum of the scores for each item. The standard score was the integer portion of the product of 1.25 and the total raw score. In China, an SDS standard score $\geqslant$ 50 indicates conscious depression.

SAS: The SAS[28] is similar to the SDS with respect to its structure and evaluation method. In particular, the SAS contains 20 items that measure the subject's anxiety levels during the prior wk. Each item receives 1 of 4 responses ranging from A = never to D = very often. Responses to positively phrased questions are scored as follows: A = 1, B = 2, C = 3, and D = 4. Responses to negatively phrased questions are scored as A = 4, B = 3, C = 2, and D = 1. The standard score for these 20 items is the integer portion of the product of 1.25 and the total raw score. An SAS standard score $\geqslant$ 50 indicates conscious anxiety. Lower SAS scores indicate milder anxiety.

The SAS and the SDS are standard assessment tools that have been proven to accurately reflect patients' subjective feelings of anxiety and depression, respectively. These two questionnaires have been translated into Chinese and have been extensively utilized in China[29].

HAMD scale: The HAMD scale[30-32] was used to evaluate the depressive states of patients during the prior week. This scale is commonly utilized by clinicians to assess depressive states. The 24-item version of the HAMD scale was used in this study. This version of the scale has clear criteria and is suitable for adults with depressive symptoms. Most of the HAMD items are scored on a 5-point scale ranging from 0 to 4, with scores of 0, 1, 2, 3, and 4 indicating no, mild, moderate, severe, and extremely severe depressive symptoms, respectively. A few HAMD items are scored on a 3-point scale ranging from 0 to 2, with scores of 0, 1, and 2 indicating no, mild-moderate, and severe depressive symptoms, respectively. The final HAMD score was the sum of the scores for each item. The following criteria were used for the total HAMD score: a score $<$ 8 points indicates that a patient is normal; a score $\geqslant$ 8 points and $<$ 20 points indicates that a patient might suffer from depression; a score $\geqslant$ 20 points and $<$ 35 points indicates that a patient certainly suffers from depression; and a score $\geqslant$ 35 points indicates that a patient is severely depressed.

HAMA scale: The HAMA scale[30-32] mainly examines two categories of factors, physical and mental. A 5-point scoring system ranging from 0 points (no symptoms) to 4 points (extremely severe symptoms) is used for each item on this scale. The sum of the scores for the 14 items on the HAMA scale is the total score. Scores $<$ 7 points indicate no anxiety; scores $\geqslant$ 7 points and $<$ 14 points indicate possible anxiety; scores $\geqslant$ 14 points and $<$ 21 points indicate definite anxiety; scores $\geqslant$ 21 points and $<$ 29 points indicate definite and significant anxiety; and scores $\geqslant$ 29 points indicate possible severe anxiety.

The HAMD and HAMA scale items were scored by two doctors who assessed these items by conversing with and observing patients. After examining each patient, the two raters independently scored the items on these scales, and the average HAMD and HAMA scores produced by the two raters were then determined.

All the questionnaires were completed before and after treatment, and the pretreatment and post-treatment results were compared.

Safety assessment

The safety evaluation encompassed the following two types of safety considerations: vital signs, which included body temperature, respiration, heart rate, blood pressure, and liver and kidney function after treatment; and moxibustion abnormalities, which included skin burns, blisters, or other moxibustion-induced discomfort. All adverse events and adverse reactions were accurately recorded.

If an adverse event occurred after treatment, a necessary treatment was provided as appropriate for the circumstances of the event in question. If extremely small blisters occurred after moxibustion, no special treatment was required; in contrast, blisters with areas larger than 1 cm^2 were broken and treated with topical ointment.

Statistical analysis

Sample size: After calculation, a required sample size of 35 cases in each group was obtained; to account for possible dropout rates of up to 10%, each group should include no fewer than 39 cases.

Data analysis: The SPSS16.0 (SPSS, Chicago, IL) statistical software package was used for statistical analysis. Normally distributed measurement data are expressed as mean ± SD; non-normally distributed measurement data are expressed in terms of median and inter quartile range. The change trends of indicators were analyzed using repeated-measures analysis of variance. Between-group comparisons of changes in indicators were analyzed using factorial analysis of variance. Count data were analyzed using χ^2 tests. Non-normally distributed measurement data and ordinal data were analyzed using nonparametric rank-sum tests. $P < 0.05$ was considered statistically significant.

RESULTS

The flow chart of this investigation is presented in Figure 2.

A total of 166 patients were included in this study. However, 6 patients dropped out during the study; in

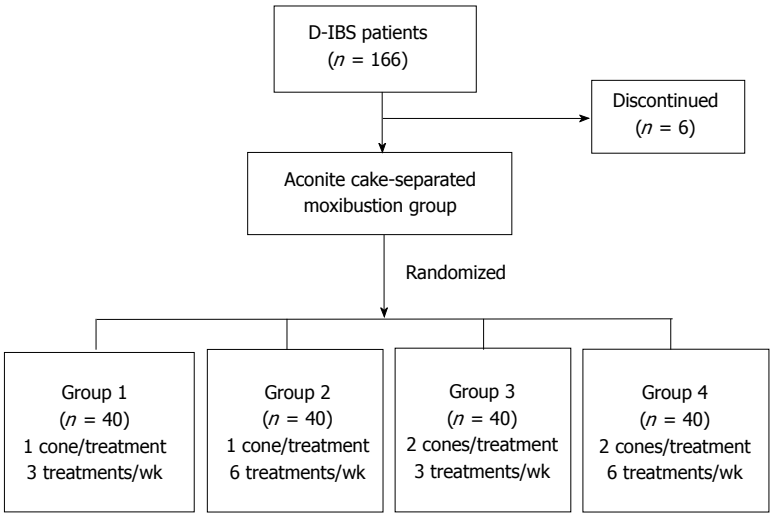

Figure 2 Flow chart overviewing the progress of patients during the course of the study. D-IBS: Diarrhea-predominant irritable bowel syndrome.

Table 1 An analysis of the general baseline conditions of the patients in each group

	Group 1	Group 2	Group 3	Group 4	*P* value
n	40	40	40	40	
Age (yr)	55	46.5	51.5	52	0.196
Gender					0.330
Male	42.5%	45%	42.5%	45%	
Female	57.5%	55%	57.5%	55%	
Disease duration (yr)	3	5	4	5	0.300
Disease severity scores	231.7 ± 68.0	221.5 ± 79.2	233.3 ± 73.9	241.5 ± 79.3	0.698

Group 1: 1 cone of moxibustion per treatment, 3 treatments/wk; Group 2: 1 cone of moxibustion per treatment, 6 treatments/wk; Group 3: 2 cones of moxibustion per treatment, 3 treatments/wk; Group 4: 2 cones of moxibustion per treatment, 6 treatments/wk.

particular, 1 case, 2 cases, 2 cases, and 1 case were lost from aconite cake-separated moxibustion groups 1, 2, 3, and 4, respectively. Thus, ultimately, each group had 40 cases that were included in the results and the statistical analysis of this investigation.

General condition of the patients

Table 1 presents comparisons of the baseline characteristics of the patients in the four randomized aconite cake-separated moxibustion groups. Comparisons of the age ($P = 0.196$), gender ($P = 0.330$), disease duration ($P = 0.300$), and disease severity score ($P = 0.582$) of the patients in these four groups revealed no significant differences, indicating that the four groups were comparable.

Post-treatment evaluations

Table 2 presents comparisons of the symptom scores at different treatment stages with the pretreatment symptom scores for the patients in each group. For the patients in all four groups, the Birmingham IBS symptom questionnaire, IBS-QOL, SDS, SAS, HAMD, and HAMA scores were significantly lower after the first and second courses of treatment than before treatment ($P < 0.001$ for all). In addition, for the patients in all four groups, the Birmingham IBS symptom questionnaire, IBS-QOL, SDS, SAS, HAMD, and HAMA scores were significantly lower after the second course of treatment than after the first course of treatment ($P < 0.001$ for all). Between-group comparisons indicated that after the second course of treatment, the Birmingham IBS symptom questionnaire score was significantly lower for group 1 (1 cone, 3 treatments/wk) and group 3 (2 cones, 3 treatments/wk) than for group 2 (1 cone, 6 treatments/wk) (5.55 ± 5.05 *vs* 10.45 ± 6.61, $P < 0.001$; 5.65 ± 4.00 *vs* 10.45 ± 6.61, $P < 0.001$). However, comparisons of quality of life scores for the patients in the 4 groups before treatment, after the first course of treatment, and after the second course of treatment revealed that at any given time point, these scores were similar across the treatment groups, with no significant between-group differences ($P > 0.05$ for all).

Table 3 presents comparisons of the degree of symptom improvement for the patients in each group at different treatment stages. After the first course of treatment, an examination of the two levels of the two tested factors of aconite cake-separated moxibustion revealed that there were significant differences between the two moxibustion frequency levels with respect to the extent of the changes in HAMA scores, with greater changes noted for the "6 treatments/wk" groups than for the "3 treatments/wk" groups ($P = 0.011$); in addition, there were interaction effects between the number of cones and moxibustion frequency ($P = 0.028$). However, after

Table 2 Comparisons of the symptom scores for patients in each group at different stages of treatment

Group	*n*		Birmingham	IBS-QOL	SDS	SAS	HAMD	HAMA
Group 1	40	Before	21.10 ± 8.62	75.75 ± 21.72	45.33 ± 11.04	42.83 ± 9.62	6.13 ± 5.43	5.90 ± 5.22
		1st course	11.60 ± 7.53[a]	60.20 ± 14.82[a]	36.20 ± 8.37[a]	36.65 ± 9.37[a]	3.75 ± 3.40[a]	4.05 ± 3.57[a]
		2nd course	5.55 ± 5.05[a,b,c]	50.65 ± 10.86[a,b]	32.43 ± 6.94[a,b]	32.08 ± 6.95[a,b]	1.75 ± 2.31[a,b]	2.23 ± 2.90[a,b]
Group 2	40	Before	23.45 ± 8.25	75.38 ± 21.02	43.45 ± 13.93	43.10 ± 12.86	7.25 ± 7.06	6.68 ± 4.74
		1st course	15.58 ± 9.15[a]	59.40 ± 18.02[a]	38.13 ± 10.54[a]	37.35 ± 10.03[a]	4.03 ± 3.28[a]	3.28 ± 3.03[a]
		2nd course	10.45 ± 6.61[a,b]	47.90 ± 10.49[a,b]	32.53 ± 7.26[a,b]	32.25 ± 7.14[a,b]	1.98 ± 2.38[a,b]	1.93 ± 2.14[a,b]
Group 3	40	Before	21.50 ± 8.82	78.53 ± 24.22	46.80 ± 13.53	46.15 ± 12.74	9.05 ± 6.54	7.53 ± 5.37
		1st course	13.03 ± 8.23[a]	58.28 ± 13.39[a]	38.40 ± 10.41[a]	37.95 ± 8.65[a]	4.60 ± 2.77[a]	3.98 ± 2.30[a]
		2nd course	5.65 ± 4.00[a,b,c]	48.83 ± 9.38[a,b]	32.50 ± 7.95[a,b]	33.70 ± 7.00[a,b]	2.10 ± 1.95[a,b]	2.20 ± 1.60[a,b]
Group 4	40	Before	23.65 ± 7.62	76.88 ± 19.90	46.00 ± 12.59	44.38 ± 12.02	7.50 ± 7.66	6.63 ± 4.78
		1st course	13.95 ± 7.69[a]	60.00 ± 16.09[a]	38.15 ± 9.76[a]	37.23 ± 10.50[a]	4.10 ± 4.89[a]	3.68 ± 2.67[a]
		2nd course	8.38 ± 6.17[a,b]	48.48 ± 13.87[a,b]	32.38 ± 9.15[a,b]	33.45 ± 8.73[a,b]	1.90 ± 3.04[a,b]	2.20 ± 2.39[a,b]

Group 1: One cone of moxibustion per treatment, three treatments/wk; Group 2: One cone of moxibustion per treatment, six treatments/wk; Group 3: Two cones of moxibustion per treatment, three treatments/wk; Group 4: Two cones of moxibustion per treatment, six treatments/wk; Before: Before treatment; 1st course: After the first course of treatment; 2nd course: After the second course of treatment; Birmingham: Birmingham irritable bowel syndrome symptom questionnaire; IBS-QOL: Irritable bowel syndrome Quality of Life Scale; SDS: Self-Rating Depression Scale; SAS: Self-Rating Anxiety Scale; HAMD: Hamilton Depression Scale; HAMA: Hamilton Anxiety Scale. Compared with the first course after treatment; [a]$P < 0.001$ *vs* before treatment; [b]$P < 0.001$ *vs* the first course after treatment; [c]$P < 0.001$ *vs* group 2.

Table 3 Comparisons of the degrees of improvement in symptoms and relevant scales for patients in each group at different stages of treatment

				Frequency		*P* value		
	Scale	*n*	Cone	Three treatments/wk	Six treatments/wk	Cone	Frequency	Conefrequency
1st course	Birmingham	40	1	42.24 ± 36.12	35.20 ± 23.84	0.643	0.845	0.254
		40	2	30.30 ± 77.95	40.25 ± 29.15			
	IBS-QOL	40	1	17.40 ± 19.04	20.25 ± 14.37	0.255	0.847	0.348
		40	2	22.63 ± 15.67	20.76 ± 13.83			
	SDS	40	1	17.99 ± 17.02	10.49 ± 9.66	0.734	0.133	0.140
		40	2	15.13 ± 19.32	15.06 ± 15.81			
	SAS	40	1	13.72 ± 13.53	11.96 ± 9.97	0.279	0.535	0.849
		40	2	15.65 ± 13.50	14.72 ± 16.67			
	HAMD	40	1	27.69 ± 48.61	39.54 ± 32.69	0.347	0.071	0.987
		40	2	33.78 ± 40.44	45.84 ± 42.93			
	HAMA	40	1	12.04 ± 65.36	46.90 ± 28.61	0.458	0.011	0.028
		40	2	33.62 ± 44.65	36.19 ± 37.95			
2nd course	Birmingham	40	1	73.74 ± 19.43	56.19 ± 21.62	0.393	0.002	0.078
		40	2	70.46 ± 27.24	65.56 ± 20.91			
	IBS-QOL	40	1	29.98 ± 16.59	34.26 ± 13.63	0.247	0.208	0.562
		40	2	34.02 ± 15.29	35.60 ± 12.88			
	SDS	40	1	26.12 ± 16.15	21.65 ± 14.35	0.149	0.373	0.373
		40	2	27.51 ± 17.52	27.51 ± 15.12			
	SAS	40	1	23.68 ± 12.83	22.54 ± 12.14	0.871	0.604	0.996
		40	2	24.02 ± 15.77	22.90 ± 13.81			
	HAMD	40	1	63.06 ± 36.74	68.88 ± 32.05	0.139	0.182	0.845
		40	2	69.63 ± 30.35	77.45 ± 29.05			
	HAMA	40	1	49.62 ± 46.44	62.61 ± 38.66	0.502	0.238	0.450
		40	2	59.19 ± 49.29	62.04 ± 32.62			

1st course: After the first course of treatment; 2nd course: After the second course of treatment; Birmingham: Birmingham irritable bowel syndrome symptom questionnaire; IBS-QOL: Irritable bowel syndrome Quality of Life Scale; SDS: Self-Rating Depression Scale; SAS: Self-Rating Anxiety Scale; HAMD: Hamilton Depression Scale; HAMA: Hamilton Anxiety Scale.

the first course of treatment, there were no significant differences between the two tested dosage levels or the two tested frequency levels with respect to the degree of improvement in the Birmingham IBS symptom questionnaire, IBS-QOL, SDS, SAS, and HAMD scores ($P > 0.05$ for all). After the second course of treatment, an examination of the two levels of the two tested factors of aconite cake-separated moxibustion revealed that there were significant differences between the two moxibustion frequency levels with respect to the extent of changes in the Birmingham IBS symptom questionnaire scores, with greater changes in the "3 treatments/wk" groups than in the "6 treatments/wk groups ($P = 0.002$); in addition, there were no interactive effects between number of cones and the moxibustion frequency ($P = 0.078$). However, after the second course of treatment, there were no significant differences between the two tested dosage levels or the two tested frequency levels with respect to the

Table 4 Correlation analysis of the degrees of improvements in relevant scales and the degrees of symptom improvement

Group	*n*	Degrees of symptom improvement (Birmingham)	Degrees of improvements in relevant scales		*r*	*P* value
Aconite cake-separated moxibustion groups	160	66.49 ± 23.25	IBS-QOL	33.46 ± 14.68	0.080	0.317
			SDS	25.70 ± 15.87	0.165	0.037
			SAS	23.28 ± 13.59	0.161	0.042
			HAMD	69.75 ± 32.28	0.088	0.276
			HAMA	58.36 ± 42.19	0.331	0.077

Birmingham: Birmingham irritable bowel syndrome symptom questionnaire; IBS-QOL: Irritable bowel syndrome Quality of Life Scale; SDS: Self-Rating Depression Scale; SAS: Self-Rating Anxiety Scale; HAMD: Hamilton Depression Scale; HAMA: Hamilton Anxiety Scale.

degree of improvement in IBS-QOL, SDS, SAS, HAMD, and HAMA scores ($P > 0.05$ for all).

Table 4 presents a correlation analysis indicating how the degrees of improvement in the scores of the utilized scales relate to the degree of improvement in symptoms. Because there were significant improvements in the scores for each scale after the second course of treatment, we performed a correlation analysis for the extent of changes in each scale and the degree of improvement in symptoms after the second course of treatment. The results of this analysis indicated that in the aconite cake-separated moxibustion groups, there were statistically significant correlations between the degree of symptom improvement and the degrees of SDS and SAS score improvement (SDS: $r = 0.165$, $P = 0.037$; SAS: $r = 0.161$, $P = 0.042$). Thus, for aconite cake-separated moxibustion treatment, the degrees of improvement in SDS and SAS scores appeared to be linearly correlated with the degrees of improvement in symptoms.

Safety assessment

Two adverse events occurred during treatment (2/160). Both of these events were mild burns during moxibustion (with one event in group 2 and the other event in group 4). No serious adverse events occurred.

DISCUSSION

In modern medicine, medication time and frequency are regulated in accordance with drug half-lives; these restrictions lead to the strict control of drug cycles. The most significant difference between moxibustion and medication is that moxibustion requires cooperation between doctors and patients. The required time and the frequency of moxibustion treatments are mainly determined by doctors' experiences. This approach will clearly result in certain errors that diminish moxibustion efficacy. Therefore, the standardization of moxibustion frequency, number of cones, and the size and weight of moxa cones, among other factors, has important significance for improving the clinical efficacy of moxibustion.

We know that effects associated with moxibustion depend largely on the thermal effects of this treatment approach[33]; in turn, the generation of these thermal effects depends on the quality of the moxibustion material, the moxa cone volume, and the number of cones applied. Aconite cake-separated moxibustion, which is an important component of moxibustion therapy, has certain distinctive characteristics. Each aspect of aconite cake-separated moxibustion may influence treatment outcomes; in particular, factors that may affect outcomes include the quality of the moxibustion material, the size of the moxa cones, the weight of the moxa cones, the aconite cake production process, the number of cones used for moxibustion, and the moxibustion frequency and duration, among other considerations. In this study, the quality of the moxibustion material, the size of the moxa cones, and the aconite cake production process were standardized. A factorial design approach was used to examine various combinations of number of cones and moxibustion frequency and thereby investigate the effects of number of cones and moxibustion frequency on the efficacy of aconite cake-separated moxibustion.

This randomized controlled trial used IBS-related scales to observe the effects of aconite cake-separated moxibustion treatment approaches involving different numbers of cone per treatment and different moxibustion frequencies on the improvement of symptoms in IBS patients. The results demonstrated that in all four treatment groups, aconite cake-separated moxibustion could significantly decrease the Birmingham IBS symptom questionnaire scores and the IBS-QOL, SDS, SAS, HAMD, and HAMA scores of IBS patients. These results indicated that for IBS patients, aconite cake-separated moxibustion treatment approaches involving different numbers of cone per treatment and different moxibustion frequencies could produce significant therapeutic effects that include improving clinical symptoms, increasing quality of life, and relieving anxiety and depression.

In addition to gastrointestinal symptoms, IBS is also closely associated with anxiety and depression[34,35]. IBS-related gastrointestinal symptoms and psychological conditions can severely affect patients' quality of life[36-38]. The survey results from a prior study[39] have indicated that populations with high anxiety and depression scores had a higher incidence of IBS compared with other populations. Investigations[40,41] have also revealed correlations among the severity of gastrointestinal symptoms, the severity of psychological conditions, and the abnormal activation of certain brain regions in IBS patients; once patients' emotional states improved, this abnormal activation of brain regions diminished. These results pro-

vided objective evidence that psychological factors influence the pathogenesis of IBS. A previous study by our research group[42] also found that in clinical practice, mild emotional stimuli can aggravate or induce gastrointestinal symptoms in IBS patients. This phenomenon occurred repeatedly; as a result, a considerable percentage of IBS patients treated in a clinical context had conditions accompanied by various degrees of anxiety, depression, and other psychological symptoms. Through brain-gut interactions, these psychological conditions can further aggravate gastrointestinal symptoms, thus creating a reciprocal causation of the physical and psychological symptoms of IBS. In this study, to examine the improvement of IBS patients' mood-related symptoms with aconite cake-separated moxibustion, we selected two types of mood-related scales to determine patients' anxiety and depression scores and their self-perceived anxiety and depression scores. The results demonstrated that IBS patients suffered from emotional disorders to various extents. In the four aconite cake-separated moxibustion groups, the average symptom, quality of life, SDS, SAS, HAMD, and HAMA scores were all significantly lower after the first and second courses of treatment than before treatment. A further correlation analysis examining how the degrees of symptom improvement related to the degrees of improvement in relevant scales revealed that improvements in SAS and SDS scores were linearly correlated with improvements in symptoms. These findings indicated that aconite cake-separated moxibustion could significantly improve emotional disorders in IBS patients and that these emotional improvements could also significantly relieve patients' IBS symptoms.

We performed statistical analyses on the symptom and quality of life scale scores and four emotion-related scales for the four groups of aconite cake-separated moxibustion patients at different time points. The results of these analyses demonstrated that the post-treatment scores significantly differed from the pretreatment scores. The scores on all scales were significantly lower after the first course of treatment than before treatment, and after the second course of treatment than after the first course of treatment, indicating that treatment duration may influence efficacy. After the end of the second course of treatment, the Birmingham IBS symptom questionnaire score was significantly lower for group 1 (1 cone, 3 treatments/wk) and group 3 (2 cones, 3 treatments/wk) compared with group 2 (1 cone, 6 treatments/wk). In particular, the "1 cone, 3 treatments/wk" group produced the lowest symptom score, suggesting that this regimen may produce the best therapeutic efficacy for the second course of aconite cake-separated moxibustion treatment.

We can draw the following conclusions from the factorial analysis results for the number of cones and moxibustion frequency. After the first course of treatment, an examination of the two levels of the two tested factors of aconite cake-separated moxibustion revealed that there were significant differences between the two moxibustion frequency levels in terms of the extent of the HAMA score changes, with greater changes observed in the "6 treatments/wk" groups than in the "3 treatments/wk" groups; in addition, there were interactive effects between the number of cones and moxibustion frequency ($P = 0.028$). After the second course of treatment, an examination of the two levels of the two tested factors of aconite cake-separated moxibustion revealed that there were significant differences between the two moxibustion frequency levels with respect to the extent of symptom score changes, with greater changes observed in the "3 treatments/wk" groups than in the "6 treatments/wk groups. These results indicated that moxibustion frequency had a significant impact on the degree of improvement in HAMA scores after the first course of treatment and the degree of symptom improvement after the second course of treatment. Interactive effects between the number of cones and the moxibustion frequency had a significant influence on the degree of HAMA score improvement. Therefore, we infer that moxibustion frequency is a key factor in symptom improvement and anxiety relief resulting from aconite cake-separated moxibustion and that the factor of number of cones has synergistic effects with moxibustion frequency for relieving anxiety. The study results demonstrated that during the first course of treatment, patient anxiety was improved more with 6 treatments/wk than with 3 treatments/wk; during the second course of treatment, patient symptoms were improved more with three treatments/wk than with six treatments/wk. We speculated that during the early stages of treatment, a higher number of moxibustion treatments could better relieve IBS patients' anxiety; as the treatment duration extended, 3 treatments/wk produced more improvement in patient conditions than 6 treatments/wk did. IBS is a chronic and recurrent functional intestinal disorder; most IBS patients require long-term treatment. Therefore, based on the comprehensive results of our study, we propose that of the examined treatment regimens, the optimal regimen for treating D-IBS with aconite cake-separated moxibustion involves one cone per treatment and three treatments per wk.

However, this study had certain limitations. Notably, better experimental results could be obtained if the study subjects could be stratified in various ways, such as by creating specific patient subgroups based on gender, disease duration, and age, or if an increased number of moxibustion frequency and dosage groups could be tested. In addition, patient follow-up after treatment is an important aspect of evaluating D-IBS treatment using aconite cake-separated moxibustion. Therefore, the aforementioned improvements to the current investigation will serve as important bases for our future research.

In summary, aconite cake-separated moxibustion is an effective and safe therapeutic approach for D-IBS. One cone per treatment and three treatments per wk is a reasonable therapeutic regimen for D-IBS. This recommended regimen can ensure therapeutic efficacy for

patients without excessively increasing the economic pressure on patients. The findings of this study provide a reliable basis for the clinical treatment of D-IBS using aconite cake-separated moxibustion.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. Clinical studies have confirmed the efficacy of herbal cake-separated moxibustion in IBS patients; however, the relationship between the quantity of stimulation with herbal cake-separated moxibustion and the resulting therapeutic efficacy has not yet been established.

Research frontiers

Herb cake-separated moxibustion is an indirect moxibustion therapy with a variety of materials. It can relieve the symptoms of abdominal pain and diarrhea, and effectively treat diarrhea-IBS (D-IBS). Therefore, factorial design was used to study the effect relationship of aconite cake-separated moxibustion in the treatment of D-IBS and to pick out a better treatment. The findings of this study provide a reliable basis for the clinical treatment of D-IBS with aconite cake-separated moxibustion.

Innovations and breakthroughs

Many previous studies on treatment of IBS with moxibustion are merely observation of efficacy. Based on moxibustion being effective for IBS, this research adopts factorial design to explore the efficacy of aconite cake-separated moxibustion in the treatment of D-IBS, as well as effects of different frequency and different cone number of aconite cake-separated moxibustion in the treatment of D-IBS, and to pick out an optimal regimen as a better treatment for D-IBS. The study provides a reliable clinical basis for future clinical treatment of D-IBS with aconite cake-separated moxibustion.

Applications

The findings of this study provide a reliable basis for the clinical treatment of D-IBS with aconite cake-separated moxibustion.

Peer review

This is a well-designed study in which authors adopt factorial design to study the effect of aconite cake-separated moxibustion in the treatment of D-IBS, and pick out an optimal regimen as a better treatment of D-IBS, and provide a reliable clinical basis for the clinical treatment of D-IBS with aconite cake-separated moxibustion.

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OBSERVATIONAL STUDY

Novel approach to identifying the hepatitis B virus pre-S deletions associated with hepatocellular carcinoma

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Abstract

AIM: To develop a novel non-sequencing method for the detection of hepatitis B virus (HBV) pre-S deletion mutants in HBV carriers.

METHODS: The entire region of HBV pre-S1 and pre-S2 was amplified by polymerase chain reaction (PCR). The size of PCR products was subsequently determined by capillary gel electrophoresis (CGE). CGE were carried out in a PACE-MDQ instrument equipped with a UV detector set at 254 nm. The samples were separated in 50 μm ID eCAP Neutral Coated Capillaries using a voltage of 6 kV for 30 min. Data acquisition and analysis were performed using the 32 Karat Software. A total of 114 DNA clones containing different sizes of the HBV pre-S gene were used to determine the accuracy of the CGE method. One hundred and fifty seven hepatocellular carcinoma (HCC) and 160 non-HCC patients were recruited into the study to assess the association between HBV pre-S deletion and HCC by using the newly-established CGE method. Nine HCC cases with HBV pre-S deletion at the diagnosis year were selected to conduct a longitudinal observation using serial serum samples collected 2-9 years prior to HCC diagnosis.

RESULTS: CGE allowed the separation of PCR products differing in size > 3 bp and was able to identify 10% of the deleted DNA in a background of wild-type DNA. The accuracy rate of CGE-based analysis was 99.1% compared with the clone sequencing results. Using this assay, pre-S deletion was more frequently found in HCC patients than in non-HCC controls (47.1% *vs* 28.1%, $P < 0.001$). Interestingly, the increased risk of HCC was mainly contributed by the short deletion of pre-S. While the deletion ≤ 99 bp was associated with a 2.971-fold increased risk of HCC (95%CI: 1.723-5.122, $P < 0.001$), large deletion (> 99 bp) did not show any association with HCC ($P = 0.918$, OR = 0.966, 95%CI: 0.501-1.863). Of the 9 patients who carried pre-S deletions at the stage of HCC, 88.9% (8/9) had deletions 2-5 years prior to HCC, while only 44.4%4 (4/9) contained such deletions 6-9 years prior to HCC.

CONCLUSION: CGE is a sensitive approach for HBV pre-S deletion analysis. Pre-S deletion, especially for short DNA fragment deletion, is a useful predictive marker for HCC.

Key words: Hepatitis B virus; Pre-S deletion; Capillary gel electrophoresis; Sequence analysis; Hepatocellular carcinoma

Core tip: Hepatitis B virus pre-S deletions are one of the risk factors associated with hepatocellular carcinoma (HCC). However, the lack of a convenient method hampers its clinical application. In this study, we develop a capillary gel electrophoresis approach that can quantitatively identify low amounts of deletions within a heterogeneous viral population. By applying this method in a case-control study, we not only confirmed previous observations that pre-S deletions increased the risk for HCC but also reported, for the first time, that only small deletions (≤ 99 bp) have a significant association with HCC, rather than large deletions.

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INTRODUCTION

Hepatitis B virus (HBV) infection plays a major causative role in the development of hepatocellular carcinoma (HCC)[1]. HBV is a small enveloped 3.2 kb DNA virus with four open reading frames (ORFs). HBV envelope proteins are encoded by three different in-frame start codons. Depending on the translated initiation site among S, pre-S1, or pre-S2, three different sized proteins are produced: a small hepatitis B surface protein (small HBs), a medium hepatitis B surface protein (middle HBs), and a large hepatitis B surface protein (large HBs)[2]. The pre-S1 region mediates the attachment of the virus to hepatocytes[3]. HBV pre-S deletion, as well as C1653T, T1753V, and A1762T/G1764A in the basal core promoter /enhancer Ⅱ region[4-6] and G1899A[4,7] in the pre-C gene, have been reported to increase the risk of HCC. Pre-S mutations are highly prevalent in countries where HBV infection is endemic[8]. In the past few decades, many studies have demonstrated that the presence of pre-S deletions are significantly associated with the development of HCC[5,9-15]. A meta-analysis that included a total of 16745 HBV-infected cases revealed that pre-S1 and pre-S2 deletions had a summary odds ratio (OR) of 2.94 (95%CI: 2.22-3.89) and 3.02 (95%CI: 2.03-4.50) for HCC, respectively[16].

Several hypotheses have been proposed to explain the pathogenic effects of HBV pre-S deletion mutants. Because the pre-S region contains several epitopes for T or B cells, HBV mutants carrying pre-S deletions can escape host immune surveillance and cause persistent infections[17]. Large HBs with deletions in the pre-S region are found to be retained in the endoplasmic reticulum (ER), resulting in ER stress, which in turn induces oxidative DNA damage and genomic instability[18,19]. Meanwhile, mutant large HBs can regulate the expression of many cellular genes, including cyclooxygenase-2, cyclin A[20], p27 (Kip1)[21-23], Bcl-2, Bcl-xL, and Mcl-1[24], thus promoting hepatocyte proliferation and inhibiting cell death induced by 5-fluorouracil. In transgenic mice, pre-S deletion mutants induced dysplasia of hepatocytes and led to the development of HCC, further supporting the fact that this variant has striking oncogenic properties[20].

To detect pre-S deletions, the most common method used previously was direct sequencing of polymerase chain reaction (PCR) products[9-15,25,26]. However, most pre-S deletion mutants co-exist with wild-type viruses in the blood of HBV carriers[27,28]. Therefore, to reveal the minority mutants in the viral population, a cloning procedure is required before sequence analysis. This methodology makes the detection of pre-S deletions both tedious and expensive, which is not suitable for large-scale screening tests. Other assays available to identify HBV pre-S deletions include polyacrylamide gel electrophoresis (PAGE)[27] and pre-S gene chip[28]. However, these techniques are relatively laborious and yield low-resolution results[29,30]. Therefore, there is an urgent need for developing a convenient and reliable method for detecting pre-S deletions.

Capillary gel electrophoresis (CGE) is a separation method based on using soluble polymers to create a replaceable molecular sieve that allows for size-based separation. This method is characterized by high sensitivity, a short analysis time, and a small sample size requirement. These features have led to many applications of CGE in analytical chemistry, pharmaceutical analysis, endocrinology, and immunology[31-34]. This method has also been successfully applied to the analysis of nucleic acids, including rapid separation of DNA restriction fragments[35] and DNA sequencing[36,37]. In this study, we established a rapid method based on CGE to identify pre-S deletion mutations in HBV-infected patients. This approach has proved to be a useful tool in the molecular epidemiological study of HBV pre-S deletions and the risk of HCC.

MATERIALS AND METHODS

Samples

The HCC cases and HBV-infected non-HCC controls were obtained from a prospective cohort started in 1992 that included 807 hepatitis B surface antigen (HBsAg)-positive and 761 HBsAg-negative individuals from 7 towns in Qidong[38]. Until the end of 2012, a total of 181 HCC were diagnosed, of which 169 were from the HBsAg-positive group and 12 from HBsAg-negative group. The 157 HCC patients who showed positive PCR results for pre-S gene amplification were selected as the case group. The inclusion criteria for the non-HCC control group were: (1) HBV-infected individual without HCC; (2) positive in HBV pre-S gene amplification; and (3) age, sex, and living location matched with the subjects in the HCC group. According to analysis of

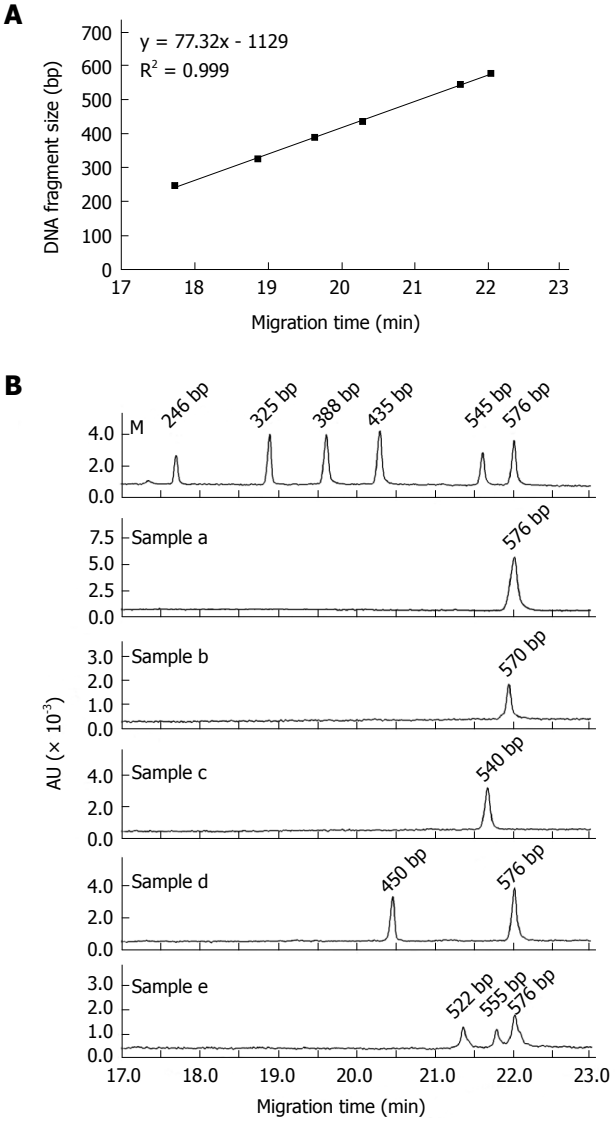

Figure 1 Establishment of a rapid method based on capillary gel electrophoresis to detect HBV pre-S deletions. A: A DNA marker was applied to construct the standard curve. CGE was used to detect the size of DNA fragments with a linear range of 246 bp to 576 bp and a regression coefficient (R^2) of 0.999; B: Representative CGE profiles of the pre-S DNA amplification products from the serum samples of 5 HBV-infected subjects. Sample a: wild-type HBV; Sample b: 6 bp deletion mutant alone; Sample c: 36 bp deletion mutant alone; Sample d: mixture of wild-type virus and a 126 bp deletion mutant; Sample e: mixture of wild-type virus and 21 bp and 54 bp deletion mutants. M: Maker (246 bp, 325 bp, 388 bp, 435 bp, 545 bp, and 576 bp); AU: Absorbance units.

the phylogenetic tree constructed by pre-C/C sequence (nt. 1824-2275), genotype C1 infection was found in 96.8% (5/157) of HCC cases and 96.2% (6/160) of the controls. The diagnosis of HCC was based on one of the following criteria: positive histological findings or positive imaging on either computerized tomography or ultrasonography along with α-fetoprotein (AFP) serum levels ≥ 400 ng/mL. The diagnosis of chronic hepatitis B (CHB) was based on the Guideline on Prevention and Treatment of Chronic Hepatitis B in China[39]. The study was approved by the local ethical committee at the Qidong Liver Cancer Institute and Shanghai Cancer Institute and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

Extraction of viral DNA and amplification of the pre-S region

The QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) was used to extract HBV DNA from 100 μL plasma according to the manufacturer's protocol. The HBV pre-S gene from nt. 2815 to 175 was amplified by semi-nested PCR using pre-SF1 [5'-GGTCACCATATTCTTGGGAAC-3' (nt. 2815-2835), forward] and pre-SR1 [5'-CTGTAACACGAGCAGGGGT-3' (nt. 184-202), reverse] as the first-round primers, and pre-SF1 and pre-SR2 [5'-CTGATGTTGTGTTCTCCATG-3' (nt. 155-175), reverse] as the second-round primers. PrimeSTAR Max polymerase (Takara, Dalian, China) was used for PCR under the following conditions: 94 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 25 s, 60 ℃ for 25 s, and 72 ℃ for 1 min, and a final extension at 72 ℃ for 7 min. The PCR products were purified using AxyPrep™ PCR Cleanup kits (Axygen Scientific, Union City, CA, United States).

Detection of pre-S deletions by CGE

Analyses of PCR samples were carried out in a PACE-MDQ instrument (Beckman Instruments, Fullerton, CA, United States) equipped with a UV detector set at 254 nm. Separations were performed in 50 μm ID eCAP Neutral Coated Capillaries (Beckman P/N 477441). The separation buffer (Beckman Coulter eCAP dsDNA 1000 kit) was injected into the capillary using 50 psi of pressure for 1 min. The samples were then injected using a voltage of 4 kV for 50 s and separated using a voltage of 6 kV for 30 min. Data acquisition and analysis were performed using the 32 Karat software (Beckman Instruments).

Detection of pre-S deletions by DNA clone sequencing

The HBV pre-S gene from nt. 2815-175 was amplified from 19 serum samples by PCR. The PCR products were cloned into the pUCm-T plasmid (Shanghai Shenergy Biocolor BioScience and Technology Co.). The recombinant plasmids were introduced into the *Escherichia coli* DH5α strain. A total of 114 clones were selected for sequencing analysis. BioEdit software (version 5.0.9; available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to align the validated sequences against the wild-type genotype C HBV DNA reference sequence (GenBank accession no. GU434374.1).

Statistical analysis

Statistical analyses were performed using the SPSS software, version 12.0 (SPSS Inc., Chicago, IL, United States). The ORs and 95%CIs were calculated using a two-by-two frequency table. The comparison between categorical variables was tested using the χ^2 test. All of the tests were two-tailed, and a P value of < 0.05 was considered to be statistically significant.

Table 1 Characteristics of the 19 cases used for clone sequencing

Cases	Sex	Age	Clinical diagnosis	Clone No.	Pre-S deletion size (bp)	Pre-S deletion site (nt)
486	Male	33	HCC	486-1, 486-2, 486-3, 486-4, 486-5, 486-6	0	/
6-200	Male	60	HCC	6-200-1,6-200-2,6-200-3,6-200-4,6-200-5,6-200-6	0	/
440	Male	52	HCC	440-1, 440-2, 440-3, 440-4, 440-5, 440-6	6	6-11
221	Male	38	HCC	221-1	0	/
				221-2, 221-3, 221-4, 221-5, 221-6	78	3107-3184
644	Male	53	HCC	644-1, 644-2, 644-3, 644-5, 644-6	0	/
				644-4	33	18-50
606	Male	41	HCC	606-1, 606-2, 606-3, 606-4, 606-6	0	/
				606-5	3	3199-3201
6-133	Male	50	HCC	6-133-1, 6-133-3, 6-133-4, 6-133-5	0	/
				6-133-2, 6-133-6	42	25-66
4-066	Female	58	HCC	4-066-1, 4-066-2, 4-066-4, 4-066-5, 4-066-6	0	/
				4-066-3	33	34-66
140	Male	64	CH	140-1, 140-2, 140-3, 140-4, 140-5, 140-6	0	/
79	Female	45	CH	79-1, 79-2, 79-3, 79-4, 79-5, 79-6	0	/
96	Male	43	CH	96-1, 96-2, 96-3, 96-4, 96-5, 96-6	0	/
141	Male	32	CH	141-1, 141-2, 141-3, 141-4, 141-5, 141-6	0	/
207	Male	60	CH	207-1, 207-2, 207-3, 207-4, 207-5, 207-6	36	62-97
259	Male	70	CH	259-1, 259-2, 259-3, 259-4, 259-5, 259-6	183	3015-3197
70	Male	49	CH	70-1, 70-5	126	2970-3095
				70-2, 70-3, 70-4, 70-6	0	/
296	Male	56	CH	296-1, 296-4, 296-5, 296-6	0	/
				296-2, 296-3	123	2922-3044
356	Male	38	CH	356-1, 356-4, 356-5, 356-6	0	/
				356-2	21	33-53
				356-3	54	2906-2959
229	Male	67	CH	229-1, 229-6	15	42-56
				229-2, 229-3, 229-4, 229-5	0	/
322	Male	58	CH	322-1. 322-2, 322-3, 322-4, 322-6	0	/
				322-5	24	45-68

HCC: Hepatocellular carcinoma.

RESULTS

Establishment of a rapid method based on CGE to detect the HBV pre-S deletions

According to previous results from our group[27] and others[40-45], pre-S deletions can occur at any position within the pre-S1 and pre-S2 genes, with deletion sizes ranging from 3 bp to 321 bp. Thus, in this study, a DNA fragment from nt. 2815-175, which covers the entire pre-S1 and pre-S2 region, was PCR amplified and subsequently analyzed using CGE. Theoretically, the length of the PCR products amplified from the HBV wild-type and mutants with the maximum deletion in the pre-S region could be 576 bp and 255 bp, respectively. Therefore, a DNA marker containing molecular weights of 246 bp, 325 bp, 388 bp, 435 bp, 545 bp, and 576 bp was chosen to construct a standard curve. CGE showed a linear detection range between 246 bp and 576 bp, with a regression coefficient (R^2) of 0.999 (Figure 1A). Figure 1B demonstrates the profiles of CGE separation for the DNA marker and the 5 PCR products of the pre-S gene amplified from the serum samples of Qidong HCC patients. The size of the PCR fragment calculated according to the CGE migration time was exactly the same as that obtained from DNA sequencing. The CGE method could clearly identify PCR products differing in size by 6 bp (sample b). More importantly, CGE could quantitatively characterize the mixed HBV population by calculating the different peak areas. In sample d, wild-type HBV co-existed with 40.1% deletion mutants, whereas in sample e, the wild-type virus was mixed with 14.7% type I mutants (-21 bp) and 34.1% type II mutants (-54 bp).

Evaluation of the accuracy of CGE in the detection of HBV pre-S deletions

A total of 114 DNA clones containing different sizes of the HBV pre-S gene were used to test the accuracy of the CGE method. The characteristics of the 19 cases used for clone construction are presented in Table 1. After PCR amplification, DNA fragments from HBV nt. 2815 to nt. 175 were subjected to CGE analysis. Among the 114 pre-S clone inserts, 77 were wild-type sequences, while the remaining 37 were truncated sequences with deletions ranging in size from 3 bp to 294 bp. For these 114 samples, the CGE method was able to correctly characterize 113 DNA samples. It only failed to detect one sample with a 3 bp deletion (Table 2). The overall accuracy of the CGE approach was 99.1% (113/114). These data indicate that CGE is a sensitive method for DNA size prediction and that it can generate comparable results to DNA sequencing.

Analysis of the pre-S deletions in HCC and non-HCC patients by CGE

We used this CGE-based method to assess the associa-

Table 2 Comparison of cloning-based sequencing and capillary gel electrophoresis analysis of HBV pre-S deletions

Size of deletion (bp)	Cloning-based sequencing (No.)	Capillary gel electrophoresis[1] (No.)
Wild type	77	77
Pre-S deletion		
0-51	21	20
54-99	6	6
102-294	10	10
Total	114	113

[1]The number of clones for which the result was in agreement with that of the sequencing analysis.

Table 3 Analysis of pre-S deletions in hepatocellular carcinoma and non- hepatocellular carcinoma patients by capillary gel electrophoresis *n* (%)

Characteristics	HCC *n* = 157	Non-HCC *n* = 160	*P* value	OR (95%CI)
Age range, yr				
≤ 48	78 (49.7)	88 (55.0)	0.343	
> 48	79 (50.3)	72 (45.0)		/
Gender				
Male	136 (86.6)	133 (83.1)	0.385	/
Female	21 (13.4)	27 (16.9)		/
Mutation pattern				
Pre-S deletion alone	22 (14.0)	11 (6.9)	0.037	2.207 (1.032-4.722)
Wild type + deletion	52 (33.1)	34 (21.2)	0.017	1.835 (1.109-3.038)
Total deletions	74 (47.1)	45 (28.1)	< 0.001	2.278 (1.430-3.630)

HCC: Hepatocellular carcinoma.

Table 4 Analysis of the correlation between the size of pre-S deletion and hepatocellular carcinoma *n* (%)

Characteristics	HCC *n* = 157	Non-HCC *n* = 160	*P* value	OR (95%CI)
Wild type	83 (52.9%)	115 (71.9%)		
Pre-S deletion				
Deletion ≤ 99 bp	54 (34.4%)	24 (15.0%)	< 0.001	2.971 (1.723-5.122)
Deletion > 99 bp	20 (12.7%)	21 (13.1%)	0.918	0.966 (0.501-1.863)

tion between HBV pre-S deletions and HCC in serum samples from 157 HCC patients and 160 non-HCC controls. The pre-S deletion was more frequently found in HCC patients than in non-HCC controls (47.1% *vs* 28.1%, respectively, $P < 0.001$, Table 3). The presence of the pre-S deletion was associated with a 2.278-fold higher risk of HCC (95%CI: 1.430-3.630). It is worth pointing out that among the 119 patients who had pre-S deletion mutants, 86 (72.2%) were co-infected with wild-type viruses.

The relationship between HBV pre-S deletion size and HCC was analyzed for the first time in this study. Interestingly, while small deletions (≤ 99 bp) occurred more frequently in the HCC group than in the non-HCC group (34.4% *vs* 15.0%, respectively, $P < 0.001$), deletions larger than 99 bp showed no distribution difference between the two groups (12.7% *vs* 13.1%, respectively, $P = 0.918$, Table 4). This result highlights the importance of deletion size in HCC risk prediction.

Longitudinal observation of pre-S deletions during the development of HCC

To determine the temporal relationship between HBV pre-S deletions and HCC occurrence, we examined pre-S deletions from serial serum samples collected 2-9 years prior to HCC diagnosis. Figure 2 shows the evolution of pre-S deletion mutants during the development of HCC. Of the 9 patients who carried pre-S deletions at the stage of HCC, 88.9% (8/9) had shown deletions 2-5 years prior to HCC, suggesting it was an early predictive marker for HCC. However, for the samples collected 6-9 years prior to HCC, only 4 patients (44.4%) contained HBV pre-S deletions, indicating that these deletions were not acquired from the beginning of the infection but arose *de novo* during the progression of liver disease. We did not find the reverse mutation in any of the 9 cases (Figure 3). It is noteworthy that, for patient 372, the wild-type HBV co-existed with approximately 10% deletion mutants 6 years before the onset of HCC, indicating that CGE can quantitatively identify a small number of deletions in a heterogeneous viral population.

DISCUSSION

HBV pre-S deletions have been consistently reported as a risk factor for HCC[5,9-15]. However, the lack of an effective detection system hampers its clinical application. In this study, we developed a CGE-based approach that can quantitatively identify a small number of deletions in a heterogeneous viral population. It is rapid, convenient, and can generate a result comparable to DNA sequencing. It has potential usage in the large-scale screening of pre-S deletions in chronic hepatitis B carriers.

Most of the previous studies used PCR direct sequencing methods to reveal pre-S deletions[9-15,25,26]. However, PCR direct sequencing is only suitable for samples containing single viral populations. Indeed, most pre-S mutant-positive cases simultaneously contain wild-type viruses, and the prevalence of mixed infections could be up to 90.9%[27,28]. In this study, we found the prevalence rates of mixed infections in patients with HCC and chronic hepatitis to be 70.3% and 75.6%, respectively. Currently, for patients infected with mixed strains of HBV, a time-consuming clone sequencing method must be performed. It is of great importance to develop an easy system that can concurrently identify two or more viral strains existing in one sample. To achieve this, two non-sequencing approaches have been reported previously: (1) PAGE followed by silver staining[27]; and (2) an oligonucleotide chip hybridization system[28]. Compared with these methods, CGE has a higher resolution and simpler detection procedure; it can automatically examine 96 samples without any manual treatment and quantitatively analyze the distribution of different types of HBV

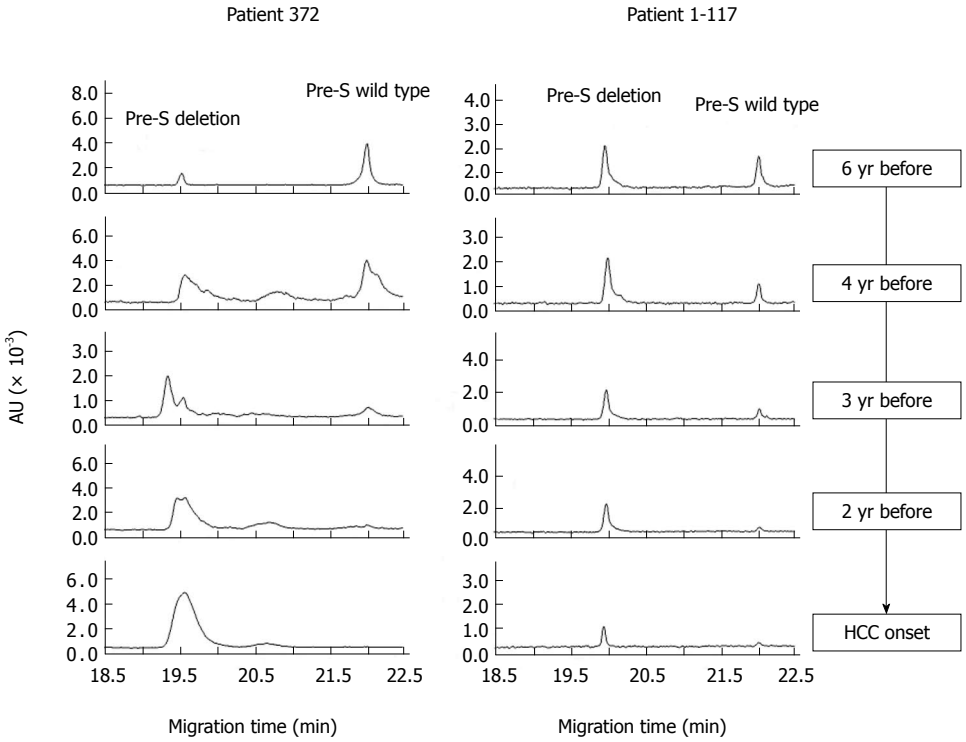

Figure 2 Longitudinal observation of hepatocellular carcinoma-related pre-S deletions during the development of hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

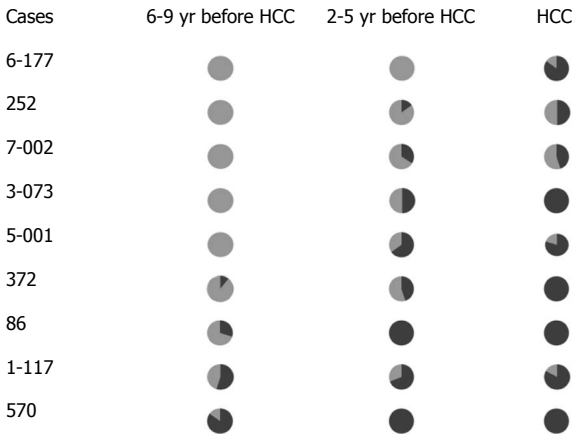

Figure 3 Longitudinal observation of hepatocellular carcinoma-related pre-S deletions in Qidong hepatocellular carcinoma patients. Black: Pre-S deletion; Grey: Wild type; HCC: Hepatocellular carcinoma.

mutants. The overall accuracy of CGE in the measurement of pre-S deletions was 99.1%. In a total of 114 sequence-known DNA clones, only one sample with a deletion of 3 bp failed to be identified by CGE. Because a 3 bp deletion of pre-S occurred in a very small number of patients, this methodological limitation should not drastically influence the overall specificity.

We applied this method to assess the HBV pre-S deletion rate in HCC and non-HCC patients. Consistent with previous studies, we found there was a significantly higher rate of pre-S deletions in HCC patients than in non-HCC patients, confirming that pre-S deletions can be used as a predictive marker for HCC (OR = 2.278; 95%CI: 1.430-3.630). Interestingly, we observed that the short stretch pre-S deletions, not the large ones, were significantly associated with HCC. While pre-S deletions ≤ 99 bp had an OR of 2.971 (95%CI: 1.723-5.122) for HCC risk, deletions > 99 bp lost their association with HCC (OR = 0.918, 95%CI: 0.501-1.863). To our knowledge, this phenomenon has never before been reported. In one recent study, Yeh *et al*[46] found that, in liver tissues, short pre-S deletions located between codon 107 and 141 (nt. 3166-52) were strongly associated with a worse postoperative prognosis. As for blood samples, several previous papers have suggested that the location of the pre-S deletions play a role in the outcome of liver disease. Pre-S deletions can occur in pre-S1, pre-S2, or in the region spanning these genes. Yeung *et al*[44] found that pre-S2 deletions, but not pre-S1, were significantly associated with the development of HCC ($P = 0.020$). Pre-S deletions generally occur in the 5' half of the pre-S2 region[43,47], and it is speculated that the small deletions in the pre-S2 region have a more significant impact on HCC development than the large deletions occurring in the pre-S1 or pre-S1+ pre-S2 region. To confirm this hypothesis, we carried out a pool analysis of the pre-S deletions in HCC and non-HCC controls by using the sequence information provided by previous studies[40-45]. The results indicated that small deletions (≤ 99 bp) have a significantly closer association with HCC than large deletions (data not shown). Thus, the CGE-based method, which only measures the size of the deletion, might have a similarly predictive value for HCC compared to the sequencing method.

Consistent with the previous reports from our group[48]

and others[14], genotype C is highly prevalent in the Qidong area. In this study, only 5 (3.2%) in the HCC group and 6 (3.8%) in the control group were found to be infected with the genotype B virus. Therefore, it is unclear from this study whether HBV pre-S deletion can be served as a predictive marker for HCC in patients infected with other viral genotypes. Pre-S deletion has been reported to occur more frequently in HBV carriers with genotype C than in those with genotype B[9,11], but very few studies have focused on the relationship between pre-S deletion and HCC in genotype B-infected patients. This intriguing issue awaits further investigation in the future.

In addition to the cross-sectional study, we also applied CGE technology to our longitudinal study. This allowed us to clearly observe the appearance and evolution of the pre-S deletion mutants during liver disease progression. The mutants seemed to have a higher survival advantage than the wild-type virus *in vivo*, as their population increased constantly until they eventually dominated the viral population. We observed no cases in which the pre-S deletion mutants were replaced by wild-type viruses. This observation strengthens the importance of early detection of pre-S deletions. Because CGE can detect the presence of pre-S deletions representing as little as 10% of the viral population, its value in HCC prediction will surpass that of the sequencing of PCR products.

Taken together, our results provided evidence to support the idea that pre-S deletions, especially short DNA fragment deletions, are a useful predictive marker for HCC. CGE is a rapid and sensitive method that can be used to detect low numbers of pre-S mutants among a mixed viral population. It will thus be a useful tool in the screening of chronic hepatitis patients who are at an increased risk for HCC.

ACKNOWLEDGMENTS

The authors wish to thank all contributors for their assistance in the Qidong prospective cohort.

COMMENTS

Background

HBV pre-S deletions have been consistently reported as a risk factor for HCC. However, the lack of an effective detection system hampers its clinical application. Most previous studies used PCR direct sequencing methods to reveal pre-S deletions. However, PCR direct sequencing is only suitable for samples containing single viral populations. Indeed, most pre-S mutant-positive cases simultaneously contained wild-type viruses. For patients infected with mixed strains of HBV, a time-consuming clone sequencing method must be performed. Thus, it is necessary to develop a sensitive, simple, and accurate method to detect HBV pre-S deletions.

Research frontiers

Various techniques have been used for the detection of HBV pre-S deletions. The capillary gel electrophoresis (CGE) method has allowed for the sensitive detection of low amounts of HBV pre-S deletion among a mixed viral population.

Innovations and breakthroughs

This is the first study aiming to develop a CGE-based approach that can quantitatively identify a small numbers of deletions within a heterogeneous viral population. By applying this method in a case-control study, we not only confirmed previous observations that pre-S deletions increased the risk of HCC, but also reported, for the first time, that only small deletions ($\leqslant$ 99 bp) have a significant association with HCC, rather than large deletions.

Applications

The CGE method developed in this study is a sensitive approach for the detection of low amounts of HBV pre-S deletion. It will be a useful tool in the screening of chronic hepatitis patients who are at an increased risk for HCC.

Terminology

CGE is a separation method based on using soluble polymers to create a replaceable molecular sieve that allows for size-based separation.

Peer review

The rapid PCR-CGE method in this study is able to accurately determine pre-S deletions associated with HCC and has greater utility than sequencing based approaches. The paper is well written.

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OBSERVATIONAL STUDY

Clinical characteristics and current management of hepatitis B and C in China

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Abstract

AIM: To describe a population of outpatients in China infected by hepatitis B virus (HBV) and/or hepatitis C virus (HCV), and assess their current management status.

METHODS: A multicenter, cross-sectional study of HBV- and/or HCV-infected patients was conducted from August to November, 2011 in western China. Patients ⩾ 18 years of age with HBV and/or HCV infections who visited outpatient departments at 10 hospitals were evaluated, whether treated or not. Data were collected on the day of visit from medical records and patient interviews.

RESULTS: A total 4010 outpatients were analyzed, including 2562 HBV-infected and 1406 HCV-infected and 42 HBV/HCV co-infected patients. The median duration of documented infection was 7.5 years in HBV-infected and 1.8 years in HCV-infected patients. Cirrhosis was the most frequent hepatic complication (12.2%), appearing in one-third of patients within 3 years prior to or at diagnosis. The HCV genotype was determined in only 10% of HCV-infected patients. Biopsy data were only available for 54 patients (1.3%). Antiviral medications had been received by 58.2% of patients with HBV infection and 66.6% with HCV infection. Nucleos(t)ide analogs were the major antiviral medications prescribed for HBV-infected patients (most commonly adefovir dipivoxil and lamivudine). Ribavirin + pegylated interferon was prescribed for two-thirds of HCV-infected patients. In the previous 12 mo, around one-fifth patients had been hospitalized due to HBV or HCV infection.

CONCLUSION: This observational, real-life study has identified some gaps between clinical practice and guideline recommendations in China. To achieve better health outcomes, several improvements, such as disease monitoring and optimizing antiviral regimens, should be made to improve disease management.

Key words: Hepatitis B; Hepatitis C; Clinical characteris-

tics; Treatment

Core tip: This observational, real-life study has identified some gaps between current clinical practice and guideline recommendations for the treatment of hepatitis B and C infections in China. To achieve better health outcomes, the findings of the study point to various improvements that need to be made to align clinical practice more closely with treatment guidelines, including: (1) routine screening for infections so that patients are diagnosed earlier; (2) more thorough evaluation of hepatitis B virus (HBV)/hepatitis C virus (HCV)-infected patients; and (3) the use of more effective antiviral agents for both anti-HBV and anti-HCV therapy and adjustment of the treatments according to the response.

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INTRODUCTION

Hepatitis B and hepatitis C caused by infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively, remain serious health problems worldwide, affecting around 2 billion people globally in the case of HBV and 130-170 million in the case of HCV[1-3]. HBV infection is potentially life-threatening and is the more serious type[1,4]. An estimated 600000 people die each year due to the acute or chronic consequences of this infection[1,5,6]. In China, hepatitis B is one of the top 3 infectious diseases reported by the Ministry of Health[7], and about 30 million people are chronically infected with HBV[8]. Every year, around 300000 people die from HBV-related diseases in China, which accounts for 40%-50% of the total HBV-related deaths worldwide[9,10].

The burden of HCV infection is also significant in China. In 1992, a nationwide survey estimated the prevalence of HCV infection in China at 3.2%[11]. Following acquisition of HCV, chronic infection develops in 75%-85% of infected individuals. Cirrhosis develops in up to 20% of those with chronic infections, and hepatocellular carcinoma (HCC) develops in 3%-4% of people with HCV-associated cirrhosis each year[12-14].

In view of the serious socioeconomic consequences of HBV and HCV infection, identifying patient characteristics and current treatment practice for these diseases will enhance regulation of their medical management. The present study was designed to provide real-life data on HBV/HCV infection in China in an effort to improve the quality of treatment and public health practice in controlling the diseases. As there are no data on the management of HBV/HCV infections in routine clinical practice in China, the objective of the study was to understand the characteristics of patients with these infections and the current treatment regimens employed, in order to identify gaps between current clinical practice and guideline recommendations[15-19].

MATERIALS AND METHODS

We undertook an observational, cross-sectional, epidemiological study at the outpatient departments of 10 hospitals in western China during the period 15 August, 2011 to 22 November, 2011. Patients with HBV and/or HCV infection who visited outpatient hepatitis departments or infectious disease physicians at the 10 participating hospitals during the study period were evaluated, whether they were currently receiving treatment or not. The study was performed in compliance with the International Conference on Harmonisation (ICH) guideline on Good Clinical Practice (GCP) and Good Pharmacoepidemiology Practice. Approval for the study was provided by either a Central Ethics Committee (Tangdu Hospital) or Ethics Committees at the hospitals where the study was performed, according to local hospital policies. Written informed consent was obtained from all participating patients.

Patients

Male or female patients who were ⩾ 18 years of age and had documented hepatitis B and/or hepatitis C infection were eligible for inclusion in the study. Hepatitis B infection was defined as a positive hepatitis B surface antigen (HBsAg) test, while hepatitis C infection was defined as a positive anti-HCV antibody test and HCV RNA test result above the limit of detection when the infection was diagnosed. Exclusion criteria included pending, inconclusive or unknown HBV or HCV test results, previous antiviral treatments for hepatitis B and/or hepatitis C that terminated > 12 mo previously, and hepatitis infection considered by the investigator as cured.

Study procedures

Investigators screened HBV/HCV outpatients continuously in their routine consultation activities at each site and enrolled eligible HBV- or HCV-infected patients. The investigators were required to complete a case report form (CRF) during the outpatient visit. Data collected from the CRFs included demographic information, vaccination history, comorbidities, diagnosis of HBV/HCV infection, concomitant infections, the most recent laboratory tests and results, the presence of hepatic complications, previous and current antiviral treatments, and other/alternative treatments taken.

As patients with HCV infection are much fewer in number than those with HBV infection, to achieve sufficient HCV patients for analysis, the investigators were requested to ensure that about one-third of the total population enrolled were HCV-infected. A total of 8361 outpatients with either HBV or HCV infection were screened for inclusion in the study, 78.0% (6524/8361) of whom had HBV infection, 21.4% (1789/8361) had HCV infection, and 0.6% (48/8361) had both. Of these,

Table 1 Demographic characteristics of the analyzed patient population with hepatitis B virus or hepatitis C virus infections *n* (%)

Characteristic	HBV (*n* = 2562)	HCV (*n* = 1406)	HBV and HCV (*n* = 42)	All patients (*n* = 4010)[1]
Age, yr (mean ± SD)	38.3 (± 12.4)	46.9 (± 13.6)	45.5 (± 11.4)	41.4 (± 13.5)
Gender				
Male	1696 (66.2)	652 (46.4)	22 (52.4)	2370 (59.1)
Female	866 (33.8)	754 (53.6)	20 (47.6)	1640 (40.9)
Ethnicity				
Han	2431(94.9)	1205 (85.7)	37 (88.1)	3673 (91.6)
Urghur	26 (1.0)	126 (9.0)	2 (4.8)	154 (3.8)
Hui	46 (1.8)	27 (1.9)	2 (4.8)	75 (1.9)
Other	59 (2.3)	48 (3.4)	1 (2.4)	108 (2.7)
Domicility				
Urban/suburban	1831 (71.5)	1212 (86.2)	33 (78.6)	3076 (76.7)
Rural	731(28.5)	194 (13.8)	9 (21.4)	934 (23.3)
Alcohol consumption	78 (3.5)	38 (2.7)	0	116 (2.9)
Medical complications	331 (12.9)	394 (28.0)	12 (28.6)	737 (18.4)
Diabetes	40 (1.6)	97 (6.9)	2 (4.8)	139 (3.5)
Liver gallstones	70 (2.7)	50 (3.6)	1 (2.4)	121 (3.0)
Alcoholic liver disease	41 (1.6)	26 (1.8)	1 (2.4)	68 (1.7)
Respiratory disease	24 (0.9)	26 (1.8)	3 (7.1)	53 (1.3)
Coronary artery disease	10 (0.4)	40 (2.8)	1 (2.4)	51 (1.3)
Hepatic complications[2]				
Hepatic cirrhosis	302 (11.8)	180 (12.8)	9 (21.4)	491 (12.2)
Jaundice	178 (7.0)	66 (4.7)	4 (9.5)	248 (6.2)
Ascites	94 (3.7)	46 (3.3)	2 (4.8)	142 (3.5)
Gastrointestinal bleeding	27 (1.1)	15 (1.1)	2 (4.8)	44 (1.1)
Hepatocellular carcinoma	11 (0.4)	13 (0.9)	1 (2.4)	25 (0.6)
Hepatic encephalopathy	4 (0.2)	11 (0.8)	1 (2.4)	16 (0.4)
HBV vaccination status				
Never	2009 (78.4)	743 (52.8)	26 (61.9)	2778 (69.3)
Childhood	122 (4.8)	136 (9.7)	3 (7.1)	261 (6.5)
Adult	43 (1.7)	149 (10.6)	0	192 (4.8)
Unknown	388 (15.2)	378 (26.9)	13 (31.0)	779 (19.4)
Pregnant	146 (5.7)[3]	47 (3.3)	2 (4.8)	195 (4.9)

[1]One patient had HDV infection; [2]Includes previous complications (3 years prior to at diagnosis) and complications occurring after diagnosis; [3]Number includes 1 patient of unknown pregnancy status.

4010 patients were enrolled in the study, including 2562 with HBV infection and 1406 with HCV infection and 42 with HBV and HCV co-infection.

Statistical analysis

Data were displayed descriptively for each patient group. Continuous variables were described as numbers, medians, minimum and maximum values, and means ± SD. Categorical variables were described as numbers and percentages for each modality. Two-sided 95%CI were calculated for prevalence data.

Data monitoring and analysis, including confirmation of eligibility and adherence to the study protocol, were conducted by an independent contract research organization and approved by the principal investigator.

RESULTS

Patient demographics and clinical characteristics of the hepatitis virus infections

A total of 4010 patients (mean age, 41.4 years; range, 18-89 years) who met the inclusion/exclusion criteria and agreed to participate were enrolled in the study (Table 1). HBV infection (without HCV) was present in 2562 patients (63.9%), HCV infection (without HBV) in 1406 (35.1%), and co-infection with both HBV and HCV in 42 (1.0%). One HBV-infected patient (0.02%) had hepatitis D virus co-infection, and 8 (0.2%) were human immunodeficiency virus (HIV)-positive, 7 of whom had HCV infection and 1 HBV infection.

Patients with HCV infection tended to be slightly older than those with HBV infection (mean, 46.9 *vs* 38.3 years) and were more commonly female (53.6% *vs* 33.8%) (Table 1). The majority of patients were of Han ethnicity (91.6%), and most lived in urban or suburban areas (76.7%). The proportion who were of Urghur ethnicity was much higher in patients with HCV infection than in those with HBV infection (9.0% *vs* 1.0%, respectively). Medical complications were present in 18.4% of the total population. The patients' vaccination histories indicated that 165 (6.4%) HBV-infected patients had received HBV vaccination, most commonly in childhood (Table 1).

The median duration of documented HBV infection was 7.5 years [range, 0-46.6 years; interquartile range (IQR), 2.7-13.8 years], which was longer than that of documented HCV infection, which was 1.8 years (range, 0-28.7 years; IQR, 6.3 mo to 4.8 years). Although around 75% of the patients had medical insurance, 87% self-paid

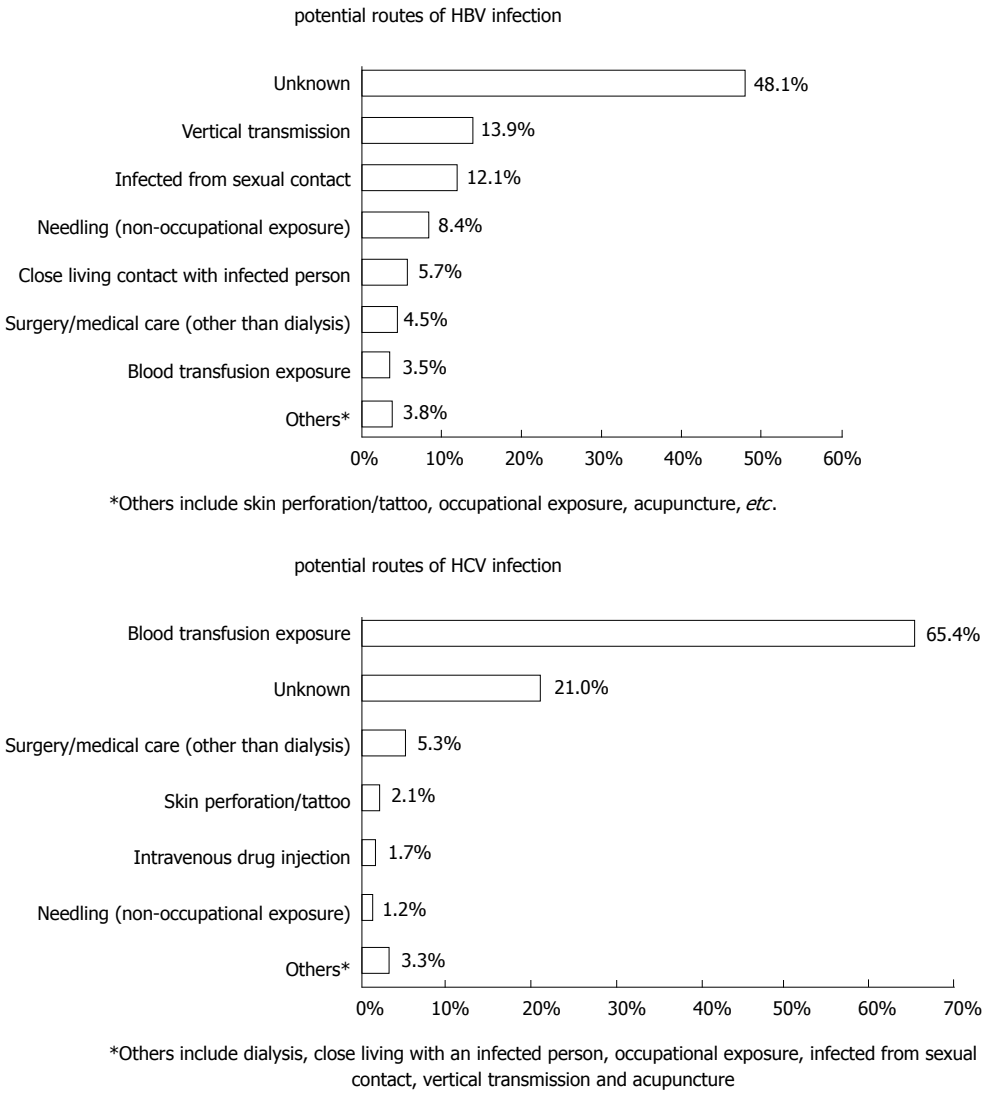

Figure 1 Most likely routes of hepatitis B virus/hepatitis C virus infection in the analyzed patient population (*n* = 4010).

to cover outpatient treatment costs.

Potential routes of infection and hepatic complications

In response to questioning regarding the most likely route of acquiring the infection, 1231 HBV-infected patients (48.1%) and 295 HCV-infected patients (21.0%) were unable to answer this question. Among patients who did answer, blood transfusion/exposure was the most likely route of infection in HCV-infected patients (65.4%), while in HBV-infected patients, vertical transmission and infection from a family or sexual contact were the most likely routes of infection (13.9% and 12.1%, respectively) (Figure 1).

Cirrhosis was the most frequent hepatic complication, presenting in 12.2% of patients overall, one-third of them within 3 years prior to or at diagnosis. Cirrhosis was slightly more common in patients with HCV infection than in those with HBV infection (12.8% *vs* 11.8%, respectively). HCC and hepatic encephalopathy were detected in 0.6% and 0.4% of patients overall (Table 1).

Laboratory assessments

Results of the most recent laboratory tests in the study population indicated that HBeAg and HBeAb were positive in 45.5% and 50.4% of HBV-infected patients with test records, respectively (Table 2); the median time from performance of these tests to the index outpatient visit was 0.5 mo (IQR, 0-4.1). More than half of the patients (55.9%) had positive HBV DNA test results in the most recent tests.

In patients with HCV infections, the most recent HCV polymerase chain reaction (PCR) test results were positive in 99.3% (1366/1375) (Table 3), and the median time from this test to the study visit was 1.3 mo. Liver function tests [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] were abnormal in one-third of HBV- or HCV-infected patients, the proportions being slightly higher in patients with HCV infection and highest in patients with HBV/HCV co-infection (Tables 2 and 3). Around 30% of patients had never had an α-fetoprotein (AFP) test. Among those who did, the

Table 2 Laboratory assessments and anti-hepatitis B virus treatments administered to patients with hepatitis B virus infection, and the responses to treatment *n* (%)

	HBV (*n* = 2562)	HBV and HCV (*n* = 42)
Laboratory assessments:		
HBsAg positive[1]	2195/2207 (99.5)	32/35 (91.4)
HBeAg positive[1]	971/2137 (45.4)	5/34 (14.7)
HBeAb positive[1]	1056/2092 (50.4)	26/32 (81.3)
HBV DNA positive[1]	1278/2286 (55.9)	313/38 (34.2)
ALT abnormal	944 (36.8)	19 (45.2)
AST abnormal	740 (28.9)	16 (38.1)
AFP abnormal	142/866 (16.3)	4/22 (18.2)
Received antiviral treatment	1490 (58.2)	22 (52.4)
Latest antiviral therapies	(*n* = 1490)	(*n* = 22)
Interferon (IFN)	109 (7.3)	2 (9.1)
PEG-IFN	99 (6.6)	14 (63.6)
Lamivudine	467 (31.3)	4 (18.2)
Telbivudine	238 (16.0)	1 (4.5)
Adefovir dipivoxil	691 (46.4)	4 (18.2)
Entecavir	230 (15.4)	2 (9.1)
Duration of completed treatment, months, median (IQR)	(*n* = 1309) 11.2 (0-187.8)	(*n* = 21) 7.8 (0.6-69.6)
Responses to treatment	(*n* = 801)	(*n* = 12)
Virological response[2]	645 (80.5)	11 (91.7)
Biochemical response[3]	465 (58.1)	5 (41.7)
Serological response[4]	105 (13.1)	2 (16.7)
Treatment status[5]		
Sustained response[6]	5/38 (13.2)	0
Virological breakthrough	20/801 (2.5)	0

[1]Patients with records from the most recent tests; [2]Includes HBV DNA not detected or below lower limit of detection, or HBV DNA decreased by ⩾ log10 IU/mL *vs* baseline; [3]ALT and AST returned to normal; [4]Includes HBeAg negative or seroconversion and HBsAg negative or seroconversion; [5]Excludes patients in whom treatment was terminated and patients not yet evaluated due to ongoing treatment; [6]Sustained response: patients who achieved and had a sustained response for at least 6 mo after completing therapy. AFP: α-Fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBeAb: Hepatitis B e antibody; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; IQR: Interquartile range; NA: Nucleos(t)ide analog; PEG-IFN: Pegylated interferon.

Table 3 Laboratory assessments and anti-hepatitis C virus treatments administered to patients with hepatitis C virus infection, and the responses to treatment *n* (%)

	HCV (*n* = 1406)	HCV and HBV (*n* = 42)
Laboratory assessments:		
HCV PCR positive[1]	1366/1375 (99.3)	38/40 (95.0)
ALT abnormal[2]	575 (40.9)	
AST abnormal[2]	499 (35.5)	
AFP abnormal	86/486 (17.7)	
Received antiviral treatment	936 (66.6)	22 (52.4)
Number of treatments	(*n* = 936)	(*n* = 23)
First	751 (80.2)	20 (87.0)
Second	140 (15.0)	3 (13.0)
Third	45 (4.8)	0
Antiviral agents used in first-time antiviral treatments	(*n* = 750)	(*n* = 20)
Interferon (IFN)	232 (30.9)	5 (25.0)
PEG-IFN	514 (68.5)	15 (75.0)
Ribavirin	702 (93.6)	15 (75.0)
Duration of completed treatment, months (median, range)	(*n* = 277) 11.8 (0.1-139)	(*n* = 3) 11.9 (5-11.9)
Responses to treatment[3]		
Quick response[4]	125/425 (29.4)	4/10 (40.0)
Early response[5]	126/349 (36.1)	2/6 (33.3)
Response at end of treatment[6]	38/276 (13.8)	2/5 (40.0)
Sustained response[7]	24/121 (19.8)	0
No response	20/276 (7.2)	0

[1]Results were unknown or unchecked for 2.2% of patients; [2]Results were unknown or unchecked for 10.8%-18.4% of patients; [3]Excludes patients in whom treatment was terminated early and patients not yet assessed due to ongoing treatment; [4]Quick response (HCV RNA negative on PCR after 4 wk of treatment): calculated in patients who had received at least 4 wk therapy; [5]Early response (HCV RNA negative on PCR negative or decreased by ⩾ 2 log10 (IU/mL) after 12 wk of treatment): calculated in patients who had received at least 12 wk therapy; [6]Response rate at end of treatment: the proportion of the patients who had received at least 24 wk therapy achieved response; [7]Sustained response: patients who had a sustained response for at least 24 wk after completing therapy. AFP: α-Fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PCR: Polymerase chain reaction; PEG-IFN: Pegylated interferon.

AFP result was abnormal in 16.9% of patients (232/1374) overall (16.3%, 17.7% and 18.2% in patients with HBV infection, HCV infection, and HBV/HCV co-infection, respectively) (Tables 2 and 3).

Genotyping

HBV DNA typing was performed in 46 HBV-infected patients (1.8%), 26 of whom (56.5%) demonstrated type B, 18 (39.1%) type C, and 2 (4.3%) type G. In HCV-infected patients, the HCV genotype was determined in 144 (10.2%), 82 of whom (56.9%) demonstrated type 1, 30 (20.8%) type 2, 20 (13.9%) type 3, and 12 (8.3%) type 6.

Ultrasound, biopsy and fibrosis assessments

Ultrasound assessments were performed relatively frequently in both HBV- and HCV-infected patients (43.1% of patients overall). Liver cancer was suspected or detected in 39 of the 1729 patients who underwent ultrasound assessments (2.3%). Slightly higher proportions of patients with HCV infection and HBV/HCV co-infection had evidence of liver cancer in comparison with patients with HBV infection (3.0% and 7.1% *vs* 1.9%, respectively).

Biopsy data were only available for 54 patients (1.3% of the study population), including 47 HBV-infected patients, 6 HCV-infected patients, and 1 with HBV/HCV co-infection. Non-invasive methods for assessing liver fibrosis, principally Fibroscan®, were employed in 13.3% of patients (534/4010).

Treatments administered and responses

Anti-HBV treatments (Table 2): At the time of the study visit, slightly over half of the patients with HBV infection (1490; 58.2%) had previously received antiviral treatments; 80.5% (1199/1490) of these patients had only ever received nucleos(t)ide analogs (NAs), while 19.5% (291/1490) had received interferon (IFN) regimens (including pegylated interferon [PEG-IFN]) or IFN + NA therapy. More than half of the treated patients had received adefovir dipivoxil (52.3%; 780/1490), while 40.1%

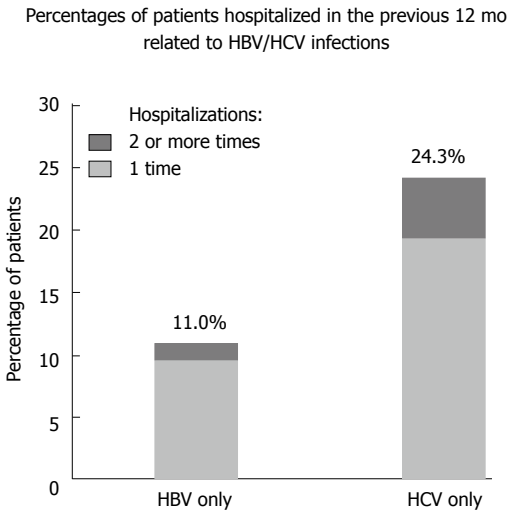

Figure 2 Percentages of patients with hepatitis B virus or hepatitis C virus infection hospitalized in the previous 12 mo related to hepatitis B virus/hepatitis C virus infections.

(598/1490) had received lamivudine.

Analysis of the most recent (176; 11.8%) or ongoing (1314; 88.2%) antiviral treatments received indicated that 86.1% (1283/1490) of those who had been prescribed anti-HBV therapy were receiving NA monotherapy, 8.9% (132) were receiving IFN monotherapy, and 5.0% (75) were receiving IFN and NA combination therapy. The most commonly used NAs were adefovir dipivoxil (46.4%) and lamivudine (31.3%) (Table 2). The median duration of treatment in 161 patients who had completed their antiviral treatment was 1 year (range, 0-8 years).

Of the 1380 patients who were prescribed anti-HBV medications at the index outpatient visit, 88.6% (n = 1222) continued on their present treatments without modification, 3.3% (n = 45) had dosage or medication changes, and 8.2% (n = 113) had anti-HBV treatment prescribed for the first time at this visit.

Treatment responses were evaluable in 801 HBV-infected patients (53.8%) who received antiviral treatment. A virological response [HBV DNA undetectable or decreased by ⩾ log10 (IU/mL) *vs* baseline] was achieved in 80.5% of these patients, a biochemical response (AST and ALT levels returned to normal) in 58.1%, and a serological response (HBeAg or HBsAg negative or seroconversion) in 13.1%. A sustained virological response was noted in only 5 of 38 patients (13.2%) who were followed for at least 6 mo after completing therapy, while relapse of HBV infection occurred in 11 of 73 patients (15.1%) who had completed treatment and were evaluated, failure of primary treatment occurred in 8 of 542 patients (1.5%) who had received antiviral therapy for at least 6 mo, and virological breakthrough occurred in 20 of 801 patients who were evaluated (2.5%).

Anti-HCV treatments (Table 3): Two-thirds of the patients with HCV infection (n = 936; 66.6%) had previously received anti-HCV therapy, the majority of whom (n = 751; 80.2%) had received 1 course of antiviral therapy, while 140 (15.0%) had received 2 courses, and 45 (4.8%) had received 3 courses. The most commonly prescribed anti-HCV agents were ribavirin + PEG-IFN or ribavirin + conventional IFN. Ribavirin + PEG-IFN was prescribed for 66.4% (498/750), 70.7% (99/140), and 73.3% (33/45) of patients for the first, second, and third treatment courses, respectively, and conventional IFN was prescribed for 30.9% (232/750), 28.6% (40/140), and 20.0% (9/45) of patients for the first, second, and third treatment courses, respectively. In patients who had completed their antiviral treatment (n = 277), the median duration of treatment was 1 year (range, 0-11.6 years).

Among 674 patients who were prescribed anti-HCV medications at the index outpatient visit, 86.5% (n = 583) continued on their present treatment without modification, 4.2% (n = 28) had dosage or medication changes, and 4.5% (n = 63) had anti-HCV treatment prescribed for the first time at this visit.

Among evaluable patients, 29.4% demonstrated a quick response (HCV RNA negative on PCR after 4 wk of therapy), 36.1% had an early response [HCV RNA negative on PCR or decreased by ⩾ 2 log10 (IU/mL) after 12 wk], and 19.8% had a sustained response after 24 wk (Table 3). However, 7.2% of patients had no response.

Other treatments

Traditional Chinese herbal medicines were taken by 40.7% of patients (1632/4010) overall (48.3% of those with HBV infection and 27.0% of those with HCV infection), while 15.8% (635/4010) took other Chinese traditional medicines (other than herbs), and 2.7% (108/4010) took vitamin preparations.

Hospitalizations

In the 12 mo prior to the study, a total of 770 patients (19.2%) had been hospitalized 1 or more times. Individuals with HCV infection or HBV/HCV co-infection were 2 or 3 times more likely to be hospitalized due to their infections than those with HBV infection (24.3% and 35.7% *vs* 11.0%, respectively). Percentages of patients who were hospitalized once and 2 or more times related to HBV/HCV infections are shown in Figure 2. The median duration of hospitalization in the total study population was 14 d (range, 2-90 d).

DISCUSSION

Despite its short-term observational nature, this cross-sectional study provides important real-time data on the clinical characteristics of hepatitis infections in outpatients and on current management practice in China. Although numerous studies of the outcome of treatments for HBV/HCV infections have been conducted in China, few studies have attempted to profile the clinical characteristics of patients with these infections or patients' acceptance of and responses to the antiviral treatments administered. Consequently, the data obtained are valuable in helping to understand how patients are currently being managed in China, and where efforts to improve the overall quality of management need to be directed.

The HBV-infected patients in this study had a longer duration of disease from the time of diagnosis (median, 7.5 *vs* 1.8 years) but a lower average age (38.3 *vs* 46.9 years) compared with the HCV-infected patients, in agreement with other studies that have generally described HCV patients as being older[20,21]. Liver cirrhosis was the most frequent complication in the patients we studied, and one-third of the cirrhosis cases were identified either before or at diagnosis, which suggests that some patients did not have a timely diagnosis. As current guidelines recommend screening for HBV infection in high prevalence countries, and screening for HCV infection in high-risk populations[17,19], establishing an effective routine screening program for HBV or HCV infections could benefit the patients' prognoses (since this is influenced by early diagnosis and treatment), and also help to reduce disease transmission.

Although the likely route of transmission was unclear in a proportion of patients, particularly those with HBV infection, the transmission routes that were recorded were similar to those reported in other studies, in that HBV infection was principally transmitted by perinatal, percutaneous and sexual exposure, whereas HCV infection was mainly caused by exposure to infectious blood[17,19]. Intravenous drug use and skin perforations/tatoos were identified as potential routes of infection in only 1.7% and 2.1% of HCV-infected patients, respectively.

The proportion of patients in whom genotyping was performed was much higher in HCV-infected patients than in HBV-infected patients (10.2% *vs* 1.8%, respectively), probably because knowledge of the HCV genotype is helpful to guide antiviral treatment, but the proportion in whom HCV genotyping was performed was significantly lower than expected. The current guideline[19] recommends that HCV RNA should be determined by a highly sensitive quantitative assay shortly before or at the initiation of treatment, and thereafter at week 12 of therapy so that treatment can be adjusted according to both the response and the individual genotype. Our findings indicate that HCV genotyping is, to some extent, neglected in clinical practice in China and that more attention needs to be paid to such testing. The predominant genotypes in the HCV-infected patients in our study were genotype 1 (56.9%) and genotype 2 (20.8%), which was similar to the findings of previous studies[22,23].

Biopsies can help to assess the degree of the liver damage and make decisions on therapy, and current guidelines recommend biopsies in patients who do not meet clear-cut guidelines for treatment[15,17,19]; however, patients are often reluctant to accept a biopsy in China because of its invasive nature, and the proportion of patients in this study who had ever received a liver biopsy was only 1.3%. In patients who do not agree to undergo a biopsy, noninvasive tests are useful in defining the presence or absence of advanced fibrosis in those with chronic hepatitis infection[19], and these tests have become more practical in clinical practice in China, although it is appreciated that non-invasive tests should not replace liver biopsies in routine clinical practice. Our data indicate that the proportion of patients who underwent non-invasive tests to assess fibrosis (principally Fibroscan®) was only 13.3%, and this test should have been more widely used.

In terms of antiviral treatment, our study showed that around 58% of HBV-infected and 67% of HCV-infected patients had received or were receiving ongoing antiviral treatment. In comparison, a study conducted in Belgium found that 25% of HBV carriers and 54% of newly diagnosed patients with HCV infection were receiving antiviral therapy[20]. In the United States, another study reported that antiviral therapy was being received by 57.9% of HBV-infected patients and 79.9% of HCV-infected patients[24].

In HBV-infected patients who were receiving antiviral treatment, nucleos(t)ide analogs (NAs) were the most commonly used antiviral agents for ongoing therapy; adefovir dipivoxil was received by 46% of patients and lamivudine by 31%. In comparison, conventional IFN or PEG-IFN was received by only 14%. The higher proportion of NA use is probably due to their greater convenience and better tolerability, which makes them more easily accepted by both patients and clinicians. However, NAs do have some shortcomings such as the requirement for life-time use and the emergence of resistance. Moreover, they have been associated with a lower HBe seroconversion rate than IFN in clinical trials[18]. Only around 54% of our HBV-infected patients who were receiving antiviral therapy were evaluable for treatment responses, but the remainder were not able to be evaluated, mainly because their treatment course was still ongoing and they hadn't reached the right time to evaluate their treatment or they might not have been evaluated on time.

On the basis of the most recent evaluation, 58% of HBV-infected patients in this study achieved normal ALT/AST levels; 80% had undetectable HBV DNA or a decrease of $\geqslant$ log10 (IU/mL), and 13% had a serological response, which are slightly lower response rates than those reported in clinical trials[18]. These lower response rates could be explained by the real-life nature of this study. Firstly, real-life populations are much more complex than those in clinical trials, and secondly, many other factors that may impact on efficacy are not as strictly controlled as in clinical trials, such as the treatment duration, dosages of antiviral agents, and adherence to treatment. In addition, adefovir dipivoxil, which had the highest usage rate in our study, has been associated with the lowest treatment response rates[18], and the proportion of patients who were receiving IFN was very low. We also noted that the sustained response rate (*i.e.*, in patients who were followed for at least 6 mo after completing their course of treatment) was only 13%, which was lower than expected, as previous reports from clinical trials have indicated that the sustained response rate at 6 mo post-treatment was 31% to 45% for PEG-IFN therapy and 17% to 31% for lamivudine monotherapy, and sustained response rates at 12 mo to 2-5 years were only slightly lower[25-27].

In HCV-infected patients, PEG-IFN + ribavirin is

recommended as the standard therapy[19]. In our study, the proportion of HCV-infected patients receiving PEG-IFN + ribavirin for first-time therapy was around 66%, and the proportion receiving conventional IFN + ribavirin was around 31%. In patients receiving treatment who were able to be evaluated, a quick response at 12 wk was achieved in 30%, an early response at 24 wk was achieved in 36%, and a sustained viral response (SVR) was achieved in only 20% of the patients who had a response at the end of treatment and were followed for at least 24 wk. In clinical trials, an SVR at 48 wk of treatment of 53% to 56% for PEG-IFN + ribavirin and 30% to 40% for conventional IFN + ribavirin has been reported[28]. The reason for the lower response rates to anti-HCV treatment in our real-life population is largely related to the fact that around one-third of the patients received conventional IFN instead of PEG-IFN because of its lower cost.

Except for the reasons cited above, we did not perform assays for HBV or HCV in this study, and this may have impacted on the quality of treatment monitoring and influenced the evaluation of treatment and the strategies employed, such as whether to discontinue treatment at appropriate time according to test results.

Around 20% of the HBV- or HCV-infected patients in our study had hepatitis-related hospitalizations within the previous 12 mo. Notably, one-fifth of HCV-infected patients who had ever been hospitalized had multiple hospitalizations in the previous year. The median duration of hospitalization was 2 wk on each occasion. Our data also showed that although 75% of the patients in the study had medical insurance, the average self-pay rate for HBV or HCV treatment in outpatient departments was 87%, which reflects the heavy burden of HBV/HCV infections to both individual patients and society.

This observational, real-life study has identified some gaps between clinical practice and guideline recommendations in China. In order to achieve better health outcomes, several improvements need to be made to align clinical practice more closely to treatment guidelines, including: (1) routine screening for HBV/HCV infections so that patients are diagnosed earlier; (2) more thorough evaluation of HBV/HCV-infected patients tests, such as implementing fibrosis evaluations, genotype testing, and biochemical assessments to guide the therapeutic strategy; and (3) considering the use of more effective or recommended agents for both anti-HBV and anti-HCV therapy and adjusting treatments according to the response.

Our study has some limitations including the fact that information on previous treatments and tests were collected from outpatient records, and some data may therefore have been missed due to incomplete or lost medical records. Also, in the overall assessment of disease management, we did not analyze the data for various subgroups of patients, and this could be undertaken in future studies.

In conclusion, the findings of this study from the western region of China provide the basis for a national longitudinal study to better characterize the clinical and treatment profiles of patients with HBV and HCV infection, and facilitate development of improved public health practice. Among the 4010 patients who were selected for inclusion in the study, analysis of disease assessment procedures and the treatments administered revealed limitations in the current management of patients with these infections that departed from current Asia-Pacific and international guideline recommendations. These limitations need to be addressed, *e.g.*, by paying more attention to the monitoring of patients, using more effective agents for treatment, and screening patients for HBV/HCV infections in order to improve health outcomes and reduce the burden of these diseases.

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COMMENTS

Background

Hepatitis B and hepatitis C caused by infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively, remain serious health problems worldwide. In China, about 30 million people are chronically infected with HBV, and around 300000 die from HBV-related diseases every year. Likewise, the burden of HCV infection is also significant in China. A nationwide survey conducted in 1992 estimated the prevalence of HCV infection at 3.2%. Following acquisition of HCV, chronic infection develops in 75%-85% of infected individuals, and in these patients, cirrhosis develops in up to 20%. In patients with HCV-associated cirrhosis, hepatocellular carcinoma develops in 3%-4% each year.

Research frontiers

In view of the major socioeconomic consequences of HBV and HCV infection, identifying patient characteristics and current treatment practice for these diseases assumes major importance for improving the quality of treatment and public health practices in controlling them. The objective of this multicenter, cross-sectional, outpatient study was to provide important information on patients with HBV or HCV infections in China to identify gaps in their management and whether treatment practices differ from Asia-Pacific and international guideline recommendations.

Innovations and breakthroughs

Despite its short-term, observational nature, the study provides important real-time data on the clinical characteristics of hepatitis infections in Chinese outpatients and on current management practice in China. Although numerous studies of the outcome of treatments for HBV/HCV infections have been conducted in China, few have attempted to profile the clinical characteristics of patients with these infections or patients' acceptance of and responses to the antiviral treatments administered. Consequently, the data obtained are valuable in helping to understand how patients are currently being managed in China, and where efforts to improve the overall quality of management need to be directed.

Applications

The study's findings point to a number of improvements that need to be made to align clinical practice more closely with current treatment guidelines for HBV and HCV infections. These include: (1) routine screening for infections so that patients are diagnosed earlier; (2) more thorough evaluation of HBV/HCV-infected patients, such as implementing fibrosis evaluations, genotype testing, and biochemical assessments to guide the therapeutic strategy; and (3) considering the use of more effective antiviral agents for both anti-HBV and anti-HCV therapy and adjusting treatments according to the response.

Peer review

This is an interesting study with a large cohort of patients.

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PROSPECTIVE STUDY

Gastric emptying evaluation by ultrasound prior colonoscopy: An easy tool following bowel preparation

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Abstract

AIM: To investigate the gastric emptying after bowel preparation to allow general anaesthesia.

METHODS: A prospective, non-comparative, and non-randomized trial was performed and registered on Eudra CT database (2011-002953-80) and on www.trial.gov (NCT01398098). All patients had a validated indication for colonoscopy and a preparation using sodium phosphate (NaP) tablets. The day of the procedure, patients took 4 tablets with 250 mL of water every 15 min, three times. The gastric volume was estimated every 15 min from computed antral surfaces and weight according to the formula of Perlas *et al* (Anesthesiology, 2009). Colonoscopy was performed within the 6 h following the last intake.

RESULTS: Thirty patients were prospectively included in the study from November 2011 to May 2012. The maximum volume of the antrum was 212 mL, achieved 15 min after the last intake. 24%, 67% and 92% of subjects had an antral volume below 20 mL at 60, 120 and 150 min, respectively. 81% of patients had a Boston score equal to 2 or 3 in each colonic segment. No adverse events leading to treatment discontinuation were reported.

CONCLUSION: Gastric volume evaluation appeared to be a simple and reliable method for the assessment of gastric emptying. Data allow considering the NaP tablets bowel preparation in the morning of the procedure and confirming that gastric emptying is achieved after two hours, allowing general anaesthesia.

Key words: Colonoscopy; Preparation; Ultrasound; Gastric emptying

Core tip: Gastric emptying have never been evaluate prior colonoscopy. An evaluation by ultrasound prior colonoscopy appeared has an easy tool to identify complete gastric emptying and will allow anesthesiologist to perform sedation.

Coriat R, Polin V, Oudjit A, Henri F, Dhooge M, Leblanc S, Delchambre C, Esch A, Tabouret T, Barret M, Prat F, Chaussade S. Gastric emptying evaluation by ultrasound prior colonoscopy: An easy tool following bowel preparation. *World J Gastroenterol* 2014; 20(37): 13591-13598 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13591.htm DOI: http://

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INTRODUCTION

Colonoscopy is the gold standard procedure for the detection of polyps and colorectal carcinoma. Bowel preparation is a key factor of high quality colonoscopy[1]. Historically, bowel-cleansing methods consisted of dietary restrictions, oral cathartics and additional cathartic enemas[2]. These preparations have been considered to be effective for colonic cleansing for colonoscopy[3]. Despite their proven efficacy, recent data identified a lack of oral intake in patients resulting in up to 10% of patients requiring repeat colonoscopy due to insufficient bowel preparation[1]. Consensus recommendations on bowel preparation have been made[4] and identified that with polyethylene glycol (PEG) up to 15% of patients did not complete the preparation because of poor palatability and/or large volumes to be ingested[5,6].

In the recent years, new colon cleansing preparations have been developed to give an alternative to 4 L of PEG. The most commonly used are osmotic laxatives, which include sodium phosphate tablets[2]. They act by increasing colon water content by attracting extracellular fluid efflux through the bowel wall. Commercially available preparations with sodium phosphate are given in two doses of tablets or bottles over at least 6-12 h. The second dose is taken at least 4 h before the colonoscopy.

Anaesthetists require the final dose to be consumed at least 4 h in advance to prevent aspiration pneumonia during general sedation[7]. In western countries colonoscopies are performed using propofol sedation which does not influence gastric emptying[8]. Fasting by reducing the residual volume of food or liquid within the stomach is thought to reduce the risk of regurgitation and aspiration of gastric contents during a procedure[7]. Meta-analysis of randomized controlled trials comparing fasting times of 2-4 h *vs* more than 4 h, report smaller gastric volumes in patients given clear liquids 2-4 h before a procedure[9]. The gastric residual volume is reduced to approximately 20 mL following a 2 h fast and is not reduced further by an additional 10 h of fasting[10]. The ASA guidelines indicate that patients should fast a minimum of 2 h for clear liquids and 6 h for a light meal prior to sedation[9]. Therefore, we explored the gastric emptying in patients over time receiving sodium phosphate tablets prior to colonoscopy.

MATERIALS AND METHODS

A prospective, non-comparative, non-randomized open label trial was performed. Full ethical approval for the study was obtained from the local institutional review board. The study was approved by an Independent Ethics Committee, (CPP "Ile-de-France III"), the French competent health authorities (the AFSSAPS, "Agence française de sécurité sanitaire du médicament et des produits de santé", which became Agence Nationale de Securité du Médicament et des Produits de Santé, ANSM) and was registered on EUDRA CT database (Eudra CT 2011-002953-80) and on www.trial.gov (NCT01398098).

Patients undergoing total colonoscopy for screening or surveillance colonoscopy from November 2011 to May 2012 were selected for inclusion in the study. Signed informed consent was always required before any study procedure. All patients included in the study were aged from 18 to 75 years, scheduled as outpatient for a colonoscopy, able to swallow tablets and presented no contraindication for sodium phosphate such as renal failure or inflammatory bowel disease. Exclusion criteria were pregnancy, allergy or hypersensitivity to the product or to one of its excipient, nausea, recurrent vomiting, abdominal pain, diabetes mellitus (insulin-dependent or non-insulin dependent) or an history of gastric surgery. The study size was limited to thirty patients to confirm the feasability of the technique.

All patients received 32 tablets of sodium phosphate bowel preparation (NaP, Colokit©) in total before colonoscopy. Each tablet contains monobasic monohydrate sodium phosphate (1102 mg) and dibasic anhydrous sodium phosphate (398 mg). Preparation was standardized as following: The day before the examination, intakes were restricted to a light, low-fibre breakfast (tea or coffee with or without sugar, light rusk-like toast, butter or similar spread, jam, marmalade or honey). After midday, only clear liquids were allowed (water, clear soup, diluted fruit juice, black tea or coffee, clear fizzy or still soft drinks). In addition, the evening before the examination, patients had to take four NaP tablets with 250 mL of water (or another clear liquid) every 15 min, repeated a further 4 times for a total of 20 tablets. On the day of the examination, patients were hospitalized in the outpatient unit for the second phase of the colon preparation with a 4 tablets of NaP intake every 15 min, in addition to 250 mL of water (or another clear liquid), repeated another two times under the same conditions, *i.e.*,12 tablets in total.

Measurement of gastric emptying was performed by antral diameters measurements using a digital abdominal ultrasound scanner as previously published[11]. Briefly, ultrasonographic views of the antrum were obtained using a sixth generation electronic convex probe (Hitachi Medical Systems, Platform Concept 5500) with transmission frequency (2.5-5 MHz) and high bandwidth (2-6 MHz). Images were obtained between peristaltic contractions with the stomach at rest prior to oral intake and every 15 min for 150 min (Figure 1). The antrum was imaged in a parasagittal plane of the epigastric area in patients in the right lateral decubitus position (Figure 2). Three measurements were performed at each time for the quantitative evaluation of the study. The cross sectional area (CSA) of the antrum was calculated according to the formula previously used by Bolondi *et al*[12] using two maximum perpendicular diameters representing the surface area of an ellipse (Figure 3). The

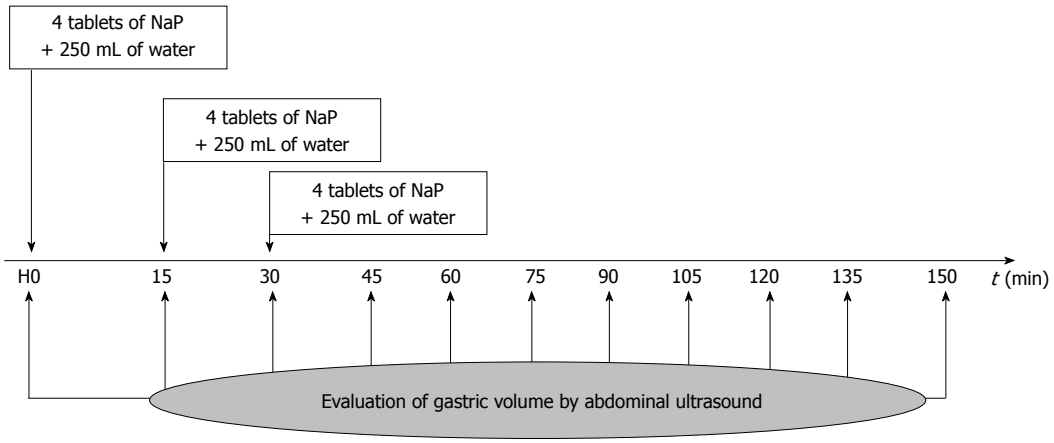

Figure 1 Protocol schedule the day of the colonoscopy. NaP: Sodium phosphate bowel preparation.

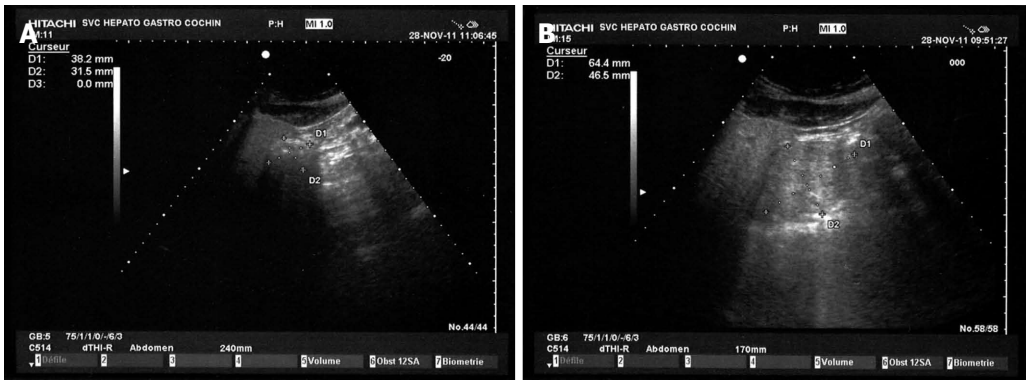

Figure 2 Measurement of the surface antral by ultrasonography in a patient in right lateral position at baseline. A: Measurement after 12 h of gastric rest; B: Measurement after the ingestion of 750 mL water plus 12 tablets of NaP in 45 min showing a distortion of the antrum. NaP: Sodium phosphate bowel preparation.

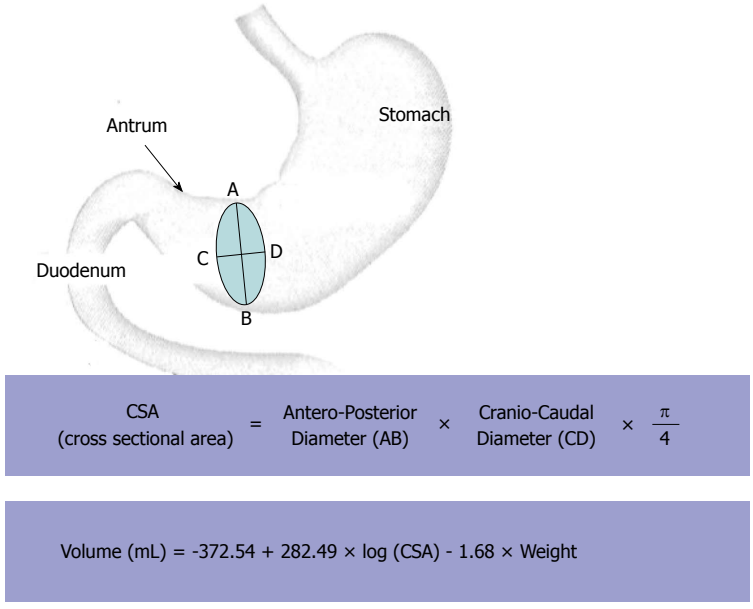

Figure 3 Ultrasound method of assessing gastric emptying time based on measurements of gastric antrum. CSA: Cross sectional area.

antral cross-sectional area is linearly correlated with the gastric volume up to 300 mL; hence it is a reliable and reproducible method to study gastric emptying[11]. The principal parameter was the percentage of subjects with antral residual volume less or equal to 20 mL, which defines an empty stomach, at different times after the

Table 1 Patients' characteristics at baseline - intention to treat population

	Total, n = 27
Age (yr)	
Median (Min - Max)	51 (31-73)
Gender	
Male/Female (%)	56/44
Weight (kg)	
Median (Min - Max)	73 (48-110)
Body Mass Index (kg/m²)	
Median (Min - Max)	25.1 (20.2-34.7)
< 18.5 kg/m²	0
18.5-25 kg/m²	13
25-30 kg/m²	13
> 30 kg/m²	1
Smoking status (%)	
Never smoke	33
Former smoker	38
Smoker	29
Preparation quality[1] (%)	
Excellent (score = 8/9)	50
Good (score = 7)	15.4
Medium (score = 6)	19.2
Insufficient (score ⩽ 5)	15.4
Caecal Intubation (%)	100
Colonoscopy results (%)	
Normal colonoscopy	14.8
Polyps	78.3
Diverticula	26.1
Ulcerations	8.7
Others anomalies	30.4
Number of detected sessile and/or Flat plans polyps which were detected	
Right colon	55
Transverse colon	11
Left colon and rectum	29

[1]Boston Bowel Preparation Scale Score.

second sequence of NaP intake.

All explorations were performed with a Fujinon EC530WM colonoscope by trained endoscopists. Endoscopies were performed using total intravenous anesthesia with propofol. A colonoscopy was defined as incomplete when there was no visualization of anatomic features, such as the ileocecal valve, appendiceal orifice, ileocolonic anastomosis or terminal ileum, as previously described[13]. The good quality of bowel preparation was defined as a global Boston Bowel Preparation Scale (BBPS) score ⩾ 7, as previously published[14].

Statistical analysis

Descriptive statistics (group size, mean, standard deviations, median, ranges, and 95%CI) were used to report patients' baseline characteristics. The analyses of primary and secondary objectives were conducted on the intention to treat (ITT) population. Comparisons between ultrasonographic examinations were performed by the Fisher exact test, the χ^2 test with Yates correction, or the Wilcoxon test when appropriate. A P value of < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

Thirty patients were included in the present study while three subjects were excluded from the ITT population (ITT, n = 27) - two subjects were not exposed to the drug and failed to complete the course of tablets. Patients' characteristics are included in Table 1. Reasons for colonoscopy included a personal history of polyps (44%), anaemia or a gastrointestinal bleeding (22%), and a family history of colorectal cancer (19%). Patients were also taking medications for diseases of the cardiovascular system (41%), the alimentary tract (22%) and the nervous system (18%). The median time between the first intake and the beginning of the second sequence was 16.3 h (range: 11.9-18.7 h). The mean time between the last intake of NaP tablets and the procedure was 4.1 ± 1.0 h.

Preparation and colonoscopy procedure

The acceptability of the preparation was high as 93% and 100% of the patients considered the intake of the 32 tablets and the total quantity of liquid easy to take, respectively. The good quality of bowel preparation, defined as BBPS score ⩾ 7, was obtained for more than 65% of the subjects and an insufficient preparation was observed in 15% (Table 1). 81% of subjects had a BBPS score ⩾ 2 in each segment. There was no need for additional cleaning for approximately two-third of subjects (70%). The mean duration of the colonoscopy was 33.1 ± 8.7 min. The mean time of withdrawal was 15.9 ± 4.4 min. All colonoscopies reached the caecum and anomalies, mainly polyps, were found in 85% of patients (Table 1).

Gastric volume ultrasound evaluation

Patients with an antral volume less than or equal to 20 mL increased from 12% at 30 min, corresponding to the last intake, to 92% at 150 min (Figure 4A). Thirty minutes after the last intake of 4 NaP tablets (60 min time), 24% of patients had an antral volume lower or equal to 20 mL. The mean antrum volume at 0 min was 18.8 ± 18.2 mL. The antral volume increased until 45 min, reaching a peak of 212.0 ± 89.1 mL, and subsequently decreasing. From 120 min (90 min after the last intake) volume values are close to the threshold value of 20 mL (Mean: 22.7 ± 21.6 mL; Figure 4B). Male subjects tended to have a 1.5 to 3 times greater antral volume than female subjects between time 0 and 90 min (Figure 5A). At 45 min, antral volume in males was significantly higher than in females (291.2 ± 87.0 mL *vs* 118.5 ± 68.8 mL, $P < 0.001$). Subjects with BMI lower or equal to 25 kg/m² tended to have a slightly greater antral volume from 45 min until 90 min than overweight subjects but off note there was no difference after 90 min (Figure 5B). Non-smokers tended to have a non-significant lower antral volume than former smokers and smokers from 0 to 90 min (Figure 5C). Residual antral volume at 120 min also tended to become lower or equal to antral volume at baseline (22.7 ± 21.6 mL *vs* 18.8

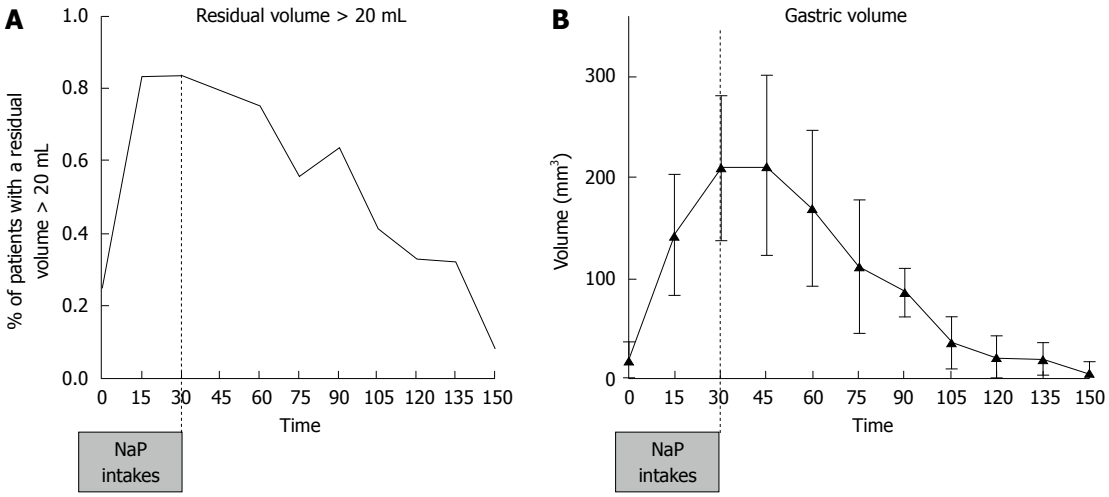

Figure 4 Evaluation of gastric volume and residual volume using ultrasonography. A: Percent of patients with a residual antral volume above 20 mL at each time. Results are expressed in percent of the overall population (n = 27); B: Modification of the gastric volume. Results are expressed in mean ± SD of the overall population (n = 27).

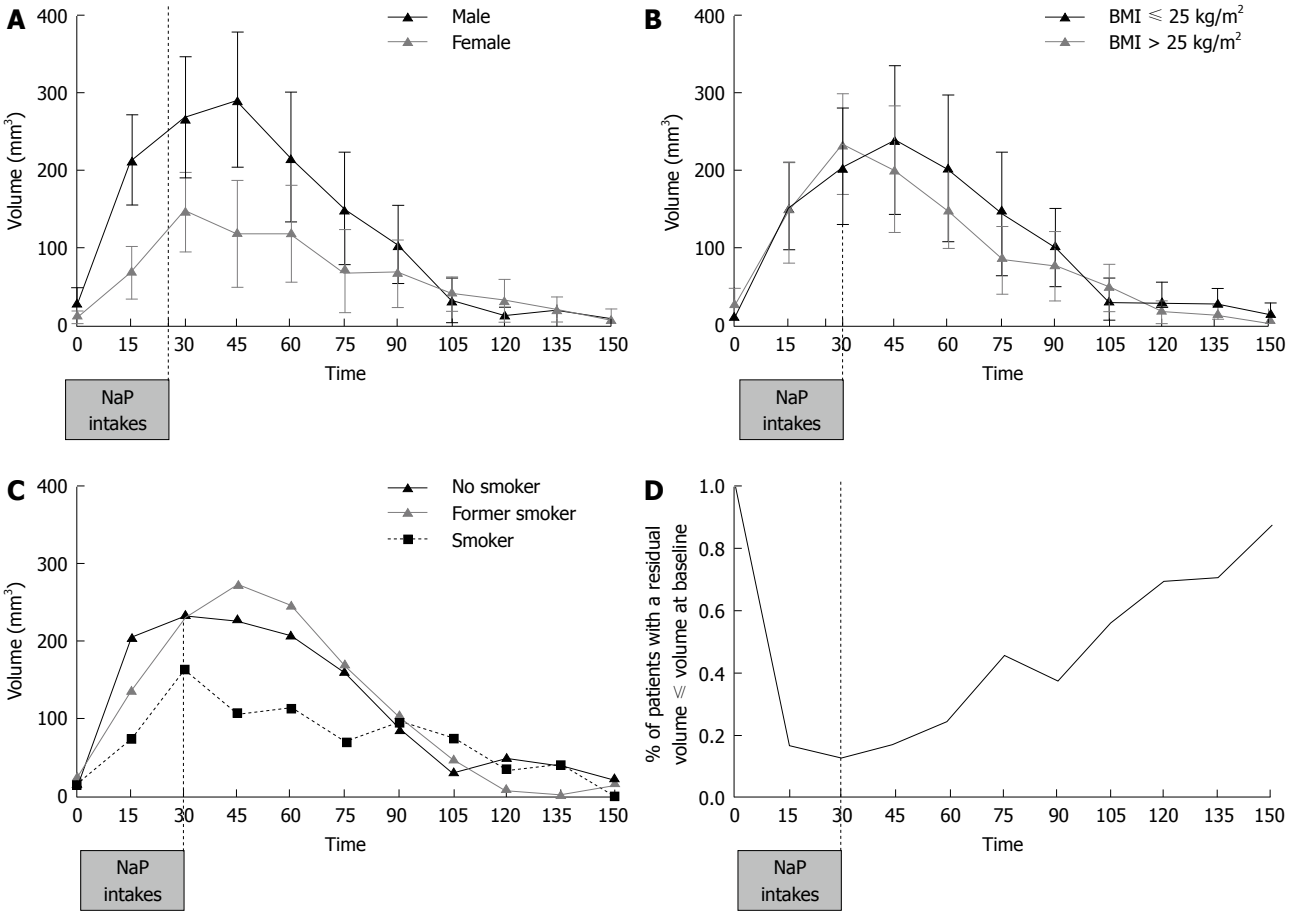

Figure 5 Evolution of gastric volume. A: Evolution of gastric volume according to gender; B: Evolution of gastric volume according to body mass index; C: Evolution of gastric volume according to or smoking habits; D: Gastric emptying evolution considering the time when gastric volume returns to baseline (after 12 h fasting).

± 18.2 mL, P = 0.15). At 105 min, more than half of patients (57%) had a residual volume lower or equal to their value at baseline. This proportion constantly increased up to 87% at 150 min (Figure 5D). Data recorded during the study did not highlight particular safety concerns, and no adverse events requiring to treatment discontinuation were reported.

DISCUSSION

Our study reports for the first time the feasibility of gastric emptying evaluation in patients undergoing colonos-

copy. The ultrasound evaluation of the antral volume will help physicians and anaesthetists to confirm the total gastric emptying and therefore reduces the risk of interstitial pneumonitis.

Colonic lavage using a specific laxative in addition to a light diet and water for 24 h on the day preceding the investigation are advised to ensure colonic cleanliness, guaranteeing quality of bowel preparation. In western countries, colonoscopy is mainly performed under general anesthesia using propofol, hence subjects should be fasting to avoid the risk of bronchial aspiration syndrome. A fasting period of at least 6 h is recommended for the ingestion of a solid meal. The ASA guidelines confirms by recommending a 6 h fasting even for light meal prior to sedation and authorizes to reduce the period to 2 h for clear liquids[9].

In addition our study confirms the efficacy of NaP tablets for colonoscopy cleansing. Gastric scintigraphy had become the reference technique for gastric emptying evaluation but has the disadvantage of irradiation, cost, limited availability of gamma cameras, the need for radioactive labels, and many sources of error. Gastric emptying assessed by scintigraphy were therefore not possible in current practice. Meanwhile, the study of Benini *et al*[15] in solid meal confirms that ultrasound is as good as scintigraphy for the measurement of gastric emptying. Perlas *et al*[11] previously identified the bedside two-dimensional ultrasonography as a useful non-invasive tool to determine gastric content and gastric volume. In their study, subjects were scanned either after 8 h of fasting, after intake of up to 500 mL plus effervescent powder, or after a standardized solid meal. Our study reinforced the feasibility of the bedside ultrasound to evaluate the gastric emptying before colonoscopy and identified a 25% gastric emptying at 60 min rising to 70% at 120 min and 87% at 150 min. Recently, the ultrasound technique was validated in patients undergoing elective surgery and reinforced the feasibility of this technique to identify the absence of residual gastric fluid at the time of anaesthetic induction[16]. Considering the lack of toxicity of the bedside ultrasound and the feasibility of the technique, ultrasound evaluation appears to be a useful technique to confirm gastric emptying prior sedation.

So far, there is no definitive standard method for the preoperative determination of gastric contents volume. Studies have shown that measuring the aspirated gastric contents to determine the gastric contents volume is an option[17]. Considering this Bouvet *et al*[18] identified that the aspirated volume of fluid gastric contents was close to the current total fluid volume in the stomach and a positive relationship between antral CSA and volume of aspirated gastric contents was made. Meanwhile, Bouvet *et al*[18] identified a better correlation between CSA and volume in patients moved to the right lateral decubitus position. In addition, Perlas *et al*[11] set the threshold of volume at 52 mL which corresponds to the upper limit of a negligible fluid volume within normal expected ranges for fasted patients. These considerations led us to study two thresholds in our study: a "severe" (20 mL) threshold value, and the broader indicated by Perlas *et al*[11]. In our study, results didn't differ from a residual value of 20 mL than 52 mL. At 45 min, 21% and 25% of patients had a residual volume below 20 mL or 52 mL, respectively. At 120 min, 67% and 87% of patients had a residual volume below 20 mL or 52 mL. An easy-to-use bedside tool to reliably assess the nature and volume of gastric contents such as ultrasound may therefore be very useful because of its simple implementation, its non-invasive and inexpensive nature.

In the present study, we identified no modification of gastric emptying between patients considering BMI and smoking activity. Patients with BMI lower or equal to 25 kg/m^2 have no significant differences of gastric emptying compare to patients with BMI above 25 (Figure 5B). In the other hand, a delayed of gastric emptying have been identify in obese patients[19]. In our study, no obese patients (BMI > 30 kg/m^2) were included and diabetes was considered as non-inclusion criteria. Those two points may explain the trend of gastric emptying in patients from 25 to 30 kg/m^2 compare to normal BMI patients. In our study, non-smokers tended to have a lower gastric volume in line with a higher gastric emptying than former smokers and smokers. It has been identified that smoking delays gastric emptying of solids, but not liquids[20]. In our study, patients' intake are pills for a total number of 32 and water, which may explain the tendency to a higher gastric emptying observed in non-smokers.

In the past decade, the colonoscopy procedure has been largely studied to improve colorectal lesions detection resulting in the identification of quality indicators for colonoscopy procedure[1]. Despite the technique required by the operator for colonoscopy, the success depends upon a number of factors including correct cecum intubation, cleaning of the colon, careful mucosal inspection, and operator experience[21]. With the rapidly rising costs of healthcare and the need to rationalize spending, it is important to spare costly repeat procedures, as in the cases of incomplete colonoscopy[1,22]. In our study all patients had a complete colonoscopy with a good overall cleansing preparation. Regarding preparations for colonic cleansing, NaP seems to have a sufficient efficacy with more than 80% of the patients with a satisfactory BBPS. In a prior study, adequate cleansing of the colon occurs in up to 80% with 4 L of PEG[1]. In an outpatient setting and in patients without contraindication to NaP tablets, NaP appears to be a feasable and effective alternative to 4 L of PEG. In addition, patients acceptability was good with up to 93% of the patients who considered the intake of the 32 NaP tablets easy to be taken. Colonoscopy demand should be expected to increase significantly in Europe over the next few years because of ongoing or planned colorectal carcinoma screening campaigns and colonoscopic surveillance program[2]. Bowel preparation is essential for both the quality of the colonoscopy and the adenoma or adenocarcinoma detection rate[1,21,23]. Aqueous sodium phosphate formulations are low volume

hyperosmotic solutions and draw water osmotically from intestinal tissues into the bowel lumen[2]. The NaP tablets are validated and commercialy available in France as in western countries for bowel preparation. Our study reinforced the use of NaP tablets for colon cleansing even in the two hours following the last intake. Our data confirm the option of using NaP tablets in patients undergoing colonoscopy in an outpatient setting.

Our study has some limitations. Firstly, the diagnostic tool extrapolates antral gastric volume from the antral area; it requires additional development and a larger validation, with independent methods to measure gastric volume. Also, the BMI of subjects in this relatively small study was measured between 20.2 and 34.7 kg/m^2, and most of the subjects were in the "normal" to "overweight" subgroups. The results may not be extrapolated to patients with obesity, as the quality of ultrasound images may be more problematic. The easy-to-use bedside ultrasound deserves an evaluation in this particular situation, as in diabetic patients. Despite those limitations, our preliminary results deserve to be validated in a larger study to confirm the usefulness of ultrasound evaluation for both the physicians and the anesthesiologists.

To conclude, gastric volume measured by extrapolation of antral ultrasound area assessment was a simple and reliable method for the assessment of gastric emptying. Respectively, 67% and 92% of subjects had gastric emptying with an antral volume below 20 mL at 120 and 150 min after the last intake of NaP tablets as bowel preparation.

COMMENTS

Background

Gastric volume evaluated by ultrasound is validated for the measurement of total gastric emptying. In the present study, we investigated the gastric emptying after bowel preparation to allow general anaesthesia.

Research frontiers

Bowel preparation is a key factor of high quality colonoscopy. Gastric emptying is necessary to allow general anaesthesia.

Innovations and breakthroughs

This study reports for the feasibility of gastric emptying evaluation in patients undergoing colonoscopy. The ultrasound evaluation of the antral volume will help physicians and anaesthetists to confirm the total gastric emptying and therefore reduces the risk of interstitial pneumonitis.

Applications

Gastric emptying evaluation using ultrasonography helps the physicians to verify the lack of bowel preparation in the stomach. Its evaluation would help both the anaesthesiologist before general anaesthesia and the gastroenterologist before colonoscopy.

Terminology

Gastric emptying is performed by antral diameters measurements using a digital abdominal ultrasound scanner. The antrum was imaged in a parasagittal plane of the epigastric area in patients in the right lateral decubitus position. Gastric emptying is correlated with the overall gastric volume.

Peer review

This is a brief prospective, non-comparative and non-randomized clinical trial. It could be published in the journal. The manuscript entitled "Gastric emptying evaluation by ultrasound prior colonoscopy: an easy tool following bowel preparation" employed thirty patients to conduct a prospective, non-comparative, and non-randomized trial and to evaluate gastric volume measured by ultrasound as the measurement of total gastric emptying. They found that gastric volume evaluation appears to be a simple and reliable method for the assessment of gastric emptying.

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Laparoscopic liver resection for malignancy: A review of the literature

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Core tip: This paper is a review article of laparoscopic liver resection for malignancy. We review all large studies that investigated the techniques and outcomes of laparoscopic liver resection as well as those comparing laparoscopic to open resections.

Abstract

AIM: To review the published literature about laparoscopic liver resection for malignancy.

METHODS: A PubMed search was performed for original published studies until June 2013 and original series containing at least 30 patients were reviewed.

RESULTS: All forms of hepatic resections have been described ranging from simple wedge resections to extended right or left hepatectomies. The usual approach is pure laparoscopic, but hand-assisted, as well as robotic approaches have been described. Most studies showed comparable results to open resection in terms of operative blood loss, postoperative morbidity and mortality. Many of them showed decreased postoperative pain, shorter hospital stays, and even lower costs. Oncological results including resection margin status and long-term survival were not inferior to open resection.

CONCLUSION: In the hands of experienced surgeons, laparoscopic liver resection for malignant lesions is safe and offers some short-term advantages over open resection. Oncologically, similar survival rates have been observed in patients treated with the laparoscopic approach when compared to their open resection counterparts.

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INTRODUCTION

Twenty-years after the first reported laparoscopic liver resection by Gagner *et al*[1], there has been an exponential growth of reports of laparoscopic liver resections but the results are yet to be fully elucidated.

Although initially described for benign and peripheral lesions, 50% of overall laparoscopic liver resections are now performed for malignant lesions[2] and a growing number of centers are now performing major resections, including right and left hepatectomies, in North America, Europe, and Asia[3-8].

The purpose of this study is to review the published literature on laparoscopic liver resection for malignant lesions. We will discuss the different types of resection, the most common as well as the innovative techniques, the surgical outcomes, postoperative complications, and oncologic results.

MATERIALS AND METHODS

A PubMed search was performed for original published studies until June 2013. Articles were selected using the indexing terms "hepatectomy", "laparoscopy", and "liver neoplasm/surgery". Keyword search for "laparoscopy", "laparoscopic", "hepatectomy", "resection", "liver cancer", "liver neoplasm", "liver tumor", "hepatic cancer", and "hepatic neoplasm". Large series with more than 30 patients with malignant lesions were included. Reported procedure other than resection, such as radiofrequency ablation and cyst fenestration were excluded.

RESULTS

About 43 studies with more than 30 patients who underwent laparoscopic resection for liver malignancy were identified. No randomized clinical trials were available. Most data were reported as case series or case-control studies. Although the first large series was in 2002, the vast majority of reports were published in and after 2009. In this review, we analyzed preoperative findings such as indications and tumor size, intraoperative findings like blood loss and operative time, short-term outcomes, as well as oncologic outcomes (Table 1).

DISCUSSION

Indications and contraindications of laparoscopic liver resection

As with open surgery, hepatocellular carcinoma (HCC) and colorectal metastasis (CRM) are the main indications of malignant tumor resection[2,3,6,9-15]. Much less commonly, resections of cholangiocarcinoma, lymphoma, and non-colorectal liver metastasis (*e.g.*, neuroendocrine tumors, breast cancer, and renal cell carcinoma) have been performed[9,13,15-18].

In patients who cannot tolerate pneumoperitonium due to their cardiopulmonary status and those with adhesions that cannot be lysed laparoscopically, laparoscopic liver resection is contraindicated. Relative contraindications are in patients with lesions adjacent to the inferior vena cava or major vessels, in whom there is a need for biliary and/or vascular reconstruction, those with hilar lesions or in those with resections that require an extensive portal lymphadenectomy[2,19,20]. Although considered a contraindication for laparoscopic resection by a large number of surgeons, successful minimally invasive approach to gallbladder cancer has been performed in 7 patients published in two papers with no reports of port site metastasis[21,22].

Minor resection

Minor resections including segmentectomy, subsegmentectomy, and wedge resections are the most common type of laparoscopic liver resection performed. Left lateral sectionectomy (LLS) which was first reported by Azagra *et al*[44], is by far the most frequently reported anatomic resection given easy visualization of the lesions with laparoscopy, peripheral location of the intended resection area, and the ease of controlling the left hepatic vein. Inagaki *et al*[45] reported 30 LLS for hepatocellular carcinoma using Hand-assisted technique. They postulated 2 advantages to the Hand-assisted technique; First, better visualization of the surgical field and the transected margin with direct manipulation by the surgeon's hand, and second, immediate hemostasis by depressing the bleeding point and proper application of hemostatic instruments.

Major resection

After the first reported cases of laparoscopic major hepatectomy by Hüscher *et al*[46] in 1998, an increasing number of series describing major resections have been reported[6,13,14,17,23,25-27,47,48].

In the largest series of major hepatectomies of 210 patients, 136 with right hepatectomoies and 74 with left hepatectomies, Dagher *et al*[3] showed a mean operative time of 250 min, with mean EBL of 300 mL. The mortality in that series was 1%.

Gumbs *et al*[49,50] reported the first laparoscopic extended left hepatectomy as well as the first laparoscopic extended right hepatectomy in 2008. The patient who underwent laparoscopic extended right hepatectomy had gallbladder adenocarcinoma and underwent preoperative portal vein embolization followed by totally laparoscopic extended right hepatic resection with preservation of segment IVa and the left half of IVb due to concerns of postoperative hepatic reserve. A total of 3 patients underwent laparoscopic extended left hepatectomy. One patient suffered a bile leak that was treated with endoscopic biliary stents.

Three large series have also included patients who underwent extended hepatectomy[14,26,29]. The papers showed that extended hepatectomy can be safely performed when undertaken by experienced surgeons and with careful patient selection.

Uncommon resections and novel approaches

While laparoscopic resection of lesions in segments 2-6, the so called "laparoscopic liver segments" have been widely performed, posterior and superior lesions have been classically unamenable to laparoscopic resection. This has changed in the last 6 years.

In a series of 300 patients, including 103 with malignant liver tumors, Koffron *et al*[48] reported 8 caudate lobectomies, 2 of which were for cancer. Cho *et al*[42] documented 10 right posterior segmentectomies in a series of 71 patients with hepatocellular carcinoma and colorectal metastasis.

Abu Hilal *et al*[51] reported 2 cases of pure laparoscopic en bloc left hemihepatectomy and caudate lobe resection for intrahepatic cholangiocarcinoma. The first patient's operative time was 360 min and blood loss was 390 mL. The second patient's operative time was 310 min and blood loss was 300 mL.

Hu *et al*[52] reported a case of multiple hepatic colorec-

Table 1 Published studies of laparoscopic liver resection for malignancy

Ref.	Year	*n*	HCC	CRM	GB CA	Cholangio	Lypmhoma	NET mets	Other	Size (cm)	EBL (mL)	Conversion	OT (min)	M	Death	LOS (d)	Margin (cm)
Choi *et al*[23]	2013	57	57	0		0	0	0	0	0.8-5.5	150-800	NA	95-380	NA	0	5 17	2.08 ± 1.68
Guerron *et al*[63]	2013	40		40						3.3	376 ± 122	5%	239 ± 17	15%	NA	3.7	1 ± 0.2
Gumbs *et al*[21]	2012	40	4	30	6	1		4	5	NA	300	6%	265	11.30%	NA	6 (1-31)	NA
Abu Hilal *et al*[13]	2012	133	18	83		2	2	17	11	0.5-14	(10-4000)	NA	30-540	0 40 %	NA	0 15	Pos 0-35%
Topal *et al*[24]	2012	81								NA	50 (10-300)	NA	120 (80-200)	11%	0	5 (3-7)	NA
Yoon *et al*[25]	2012	89	89							3 ± 1.6	NA	4.70%	2675 ± 138	NA	NA	NA	1.4 ± 1.4
Cannon *et al*[26]	2012	35		35						NA	202	NA	NA	23%	0	4.8 *vs* 8.3	Neg 97%
Ikeda *et al*[27]	2012	30	16	13		1				2.6 ± 1	146 (0-550)	0	79-697	NA	0	5-44	5 (1-30)
Dagher *et al*[28]	2010	163	163							3.6 (1-20)	250 (30-2000)	9.20%	180 (60-655)	11.60%	1.20%	7 (2-76)	12 (0-58)
Shafaee *et al*[29]	2011	66	3	55			1	3	2	0.5-12.5	300 (0-5000)	11%	180 (80-570)	26%	0	6 (2-42)	0.3 (0-4)
Lai *et al*[30]	2011	56	42	14						0.9-5.4	175 (5-2000)	3.60%	150 (75-307)	14.30%	0	6.5 (2-13)	89.3% R0
Belli *et al*[31]	2011	65	65							3.8 (1-9)	311 ± 180	10.70%	NA	20%	1.50%	8.2 (3-15)	NA
Truant *et al*[32]	2011	36	36							2.9 ± 1.2	452 ± 442	19.40%	NA	25	0	NA	0.95
Nguyen *et al*[43]	2011	108								3.2	101	NA	256	NA	NA	NA	1.12, 1.5
Lee *et al*[33]	2011	31	31							2.5 (1.5-9)	150 (10-1610)	18.20%	225 (100-420)	6.10%	0	5 (2-15)	1.8 (0-4)
Hu *et al*[34]	2011	30	30							6.7 ± 3.1	520 ± 30	NA	180 ± 45	13.30%	NA	13 ± 2.1	NA
Kazaryan *et al*[35]	2010	107		107						3 (0.5-12)	300 (50-4000)	4.20%	188 (64-488)	NA	0.90%	3 (1-42)	NA
Martin *et al*[5]	2010	65	14%	48%		12%			26%	4 (2-15)	150 (20-1000)	4%	140 (50-240)	23%	1%	3 (1-13)	Pos 3%
Zhang *et al*[41]	2010	70	29	28		2	1		10	NA		8.80%	150 (20-390)	14.80%	0	6 (1-20)	Neg 100%
Tranchart *et al*[36]	2010	42	42							3.58 ± 1.75	346 ± 435.7	NA	233.1 ± 92.7	20.40%	2.40%	6.7 ± 5.9	1.04 ± 0.8
Yoon *et al*[8]	2010	69	69							3.1 ± 1.5		7.20%	280.9 ± 128.2	21.70%	NA	9.9 ± 5.6	1.5 ± 1.6
Nguyen *et al*[62]	2009	109		109						NA	200 (20-2500)	3.70%	234 (60-555)	12.00%	NA	4 (1-22)	Neg 94.4%
Sasaki *et al*[37]	2009	72	37	35						2.5 (1.5-8.5)	64 (1-917)	NA	177 (70-430)	5.50%	NA	8.5-10	0.6 (0-4)
Bryant *et al*[16]	2009	100	64	22		3			11	NA	(0-2000)	9.60%	30-480	15.10%	0	7.9 (2-76)	1.53 (0-5.8)
Inagaki *et al*[38]	2009	52	36	10		3			3	3.1 ± 1.8	393 ± 564	2.90%	214 ± 93	10%	NA	17	NA
Cho *et al*[4]	2009	35	27	8						3.4 (1.5-7.5)	619.5 (20-1800)	NA	299.9 (80-630)	27.50%	NA	10.9 (5-38)	1.3 (0.2-5)
Huang *et al*[39]	2009	33	27	3		1			2	3.4 (1-11)	200 (100-400)	6.70%	120 (90-177.5)	11.11%	NA	5	0.2-3.2
Otsuka *et al*[40]	2009	76	55	16		2			3	3.68 ± 2.8	262 ± 344.8	NA	268 ± 123.1	11%	0	11.8 ± 7.3	NA
Castaing *et al*[14]	2009	60		60						3.3 ± 1.1	NA	10%	NA	27%	1.70%	10 (5-50)	Neg 87%
Dagher *et al*[3]	2009	210								NA	300 ± 391	12%	250 ± 103.8	22%	1.00%	6 ± 3.5	10.5 ± 13.6
Zhang *et al*[41]	2009	49	33	10		6			3	6.2 (0.8-15)	280 (10-1000)	NA	NA	NA	0	NA	1.2 (0.5-6)
Cho *et al*[42]	2008	71	52	19						3.7 ± 3	425 (20-900)	5%	240 (30-540)	15%	0	9 (4-21)	1 (0.3-6.8)
Topal *et al*[6]	2008	77								3.8 (0.5-17)	500 (10-7000)	9%	95 (30-385)	5.50%	NA	6 (0-41)	Pos in 1.2%
Chen *et al*[53]	2008	116	116							1.3-5.1	101.6-667.2	NA	118.4-488.7	6%	NA	6	NA
Buell *et al*[17]	2008	106	36	31		8			31	3.1-6.4		NA	NA	NA	3.70%	NA	2.80%
Hompes *et al*[22]	2007	45	8	32	1	4		1	1	4 (0.8-17)	200 (5-4000)	6.60%	115 (45-360)	24%	0	7 (3-41)	1 (0.1-3)
Dagher *et al*[47]	2007	38	24	9		3			2	3.8 (2.2-8)	397 (100-2300)	10%	227 ± 109	16%	1.40%	5.9 ± 5.6	1.1 (0.1-3.2)
Koffron *et al*[48]	2007	103								NA	102	6% to ha	99	9.30%	0	NA	NA
Vibert *et al*[9]	2006	65	16	41		2		3	3	0.8-18	NA	13%	85-515	19%	1.10%	11 (3-47)	NA
Mala *et al*[10]	2005	47	1	42				1	3	3 (0.8-15)	700 (< 100-5000)	6%	187 (80-334)	16%	0	3.5 (1-14)	Pos in 6%
O'Rourke *et al*[7]	2004	33		22					11	NA	20-2000	9.40%	40-370	14%	0	NA	1.1 (0-3)
Inagaki *et al*[45]	2003	52	36							3.1 (1-14)	393 ± 564 (5-3500)	2.90%	214 (61-375)	26.40%	0	14.8	NA
Gigot *et al*[59]	2002	37	10	12				5	10	3.3 (1-6)	NA	10.80%	NA	21%	0	7 (2-16)	Pos in 6.7%

HCC: Hepatocellular carcinoma; CRM: Colorectal metastasis; NET mets: Neuroendocrine metastasis; GB CA: Gallbladder adenocarcinoma; Cholangio: Cholangiocarcinoma; NA: Unavailable; EBL: Estimated blood loss; OT: Operative time; LOS: Length of stay; Margin: Resection margin size; Pos: Positive margin rate; R0: R0 resection rate.

tal metastases of the left lateral and right posterior segments who underwent laparoscopic partial hepatectomy of the right posterior segment using a retroperitoneal approach, followed by laparoscopic hepatic left-lateral segmentectomy using a transabdominal approach. The operative time was 120 min, blood loss volume was 150 mL and the patient was discharged on postoperative day 9.

Hand-assisted technique

Hand-assisted technique entails the creation of a 7-8 cm incision that would allow the operating surgeons' hand access to the abdominal cavity through a gas-tight port. The theoretical advantages of the hand-assisted technique is the ability to apply liver traction and mobilization, palpate the liver with tactile feedback, and help achieve hemostasis by applying compression[17,45,48].

The Hand-assisted technique has been proposed to provide a safer and more attainable approach to laparoscopic resection[17,48]. The application of the hand-assisted technique has been proposed to help with difficult resections like those involving the posterior-superior segments[53]. In their published series in 2007, Koffron *et al*[48] reported 103 patients with laparoscopic liver resection. In 6% of the patients a conversion from a totally laparoscopic to hand-assisted surgery was necessary while the conversion to open surgery was nil. They suggested that the use of hand-assistance may help prevent conversion to open surgery. However, there is no consensus on the usefulness of the hand-assisted technique as other authors have doubted its usefulness[7,54].

Robotic resection

Robotic laparoscopic resections are now well reported in the literature. While the technique of port placement is different from laparoscopic surgery, most studies have showed encouraging results with comparable results to laparoscopic approach[55]. In a series of 70 patients, 42 of which had malignant lesions, Giulianotti *et al*[56] reported 4 conversions to open surgery (5.7%), a median operative time for a major resection of 313 min and 198 min for minor resection, median blood loss of 150 mL for minor resection and 300 mL for major resection. The rate of complications was 21%, while the mortality rate was 0%.

In a matched analysis study, Daouadi *et al*[57] showed that patients undergoing robotic liver resection had longer operative times when compared to laparoscopic resection (253 min *vs* 199 min), however, the robotic approach increased the percentage of major hepatectomies that were completed in a purely minimally invasive approach. There was no difference between the two groups in term of other surgical outcomes like blood loss, resection margin, and 3-mo mortality.

Operative technical details

Precoagulation of the surface of hepatic parenchyma using a variety of instruments and techniques like the Salient monopolar Endo FB 3.0 or Endo SH 2.0 (Salient Surgical Technologies, Inc., Dover, NH, United States) combined with the Microtaze (Alfresa-Pharma Co., Inc., Osaka, Japan), the Cool-tipTM RFA system (Valleylab, Tyco Healthcare, Mansfield, Massachusetts, United States), and Radiofrequency precoagulation (Laparoscopic Habib 4 ×, Angiodynamics, Queensbury) was postulated as a way to help reduce blood loss by many authors[37,40,53].

The most commonly used equipment for parenchymal transection were the Harmonic scalpel (Ethicon Endo-Surgery), the laparoscopic ultrasonic surgical system (USU; Olympus Optical, Tokyo, Japan), and Cavitron Ultrasonic Surgical Aspirator CUSA (Valleylab)[13,30,32,37,38,58].

Other less common techniques included the use of staplers[17,30,38] and crush-clamp technique[40]. These are largely dependent on surgeon or center preference.

The Pringle maneuver in which the portal triad is clamped is the most common method used to minimize blood loss during liver resections. There was no consensus in the published literature as to the role of the Pringle maneuver in laparoscopic resections. It was used intermittently by some groups to reduce blood loss[13,30,32].

Belli *et al*[31] described the laparoscopic application of Pringle maneuver using tape that was placed around the porta hepatis by the use of Endo Retract TM Maxi (Tyco Healthcare, Norwalk, Conn., United States). This was passed through a 16-Fr rubber drain and used as a tourniquet when necessary[31]. Others, like Tranchart *et al*[58], purposely didn't use the Pringle maneuver although an explanation to this approach was not provided.

A number of studies have reported that inflow occlusion is required less often and for shorter periods during laparoscopic liver resections[5,35,48]. This was postulated to be due to the magnification and improved visualization of the hepatic parenchyma as well as the tamponade effect of pneumoperitonium on venous bleeding.

Outcomes of laparoscopic liver resection

Operative blood loss: Due to the heterogeneity of the reported cases, blood loss was highly variable in different studies. Average blood loss ranged between 50 to 700 mL. Dagher *et al*[28] and Otsuka *et al*[40] showed that with late experience, blood loss was significantly lower than in early cases, *i.e.*, surgeons had lower blood loss during laparoscopic resection with more procedure performed. Six studies have showed that laparoscopic resection was associated with lower blood loss than open surgery[5,6,24,26,48,58]. Martin *et al*[5] showed lower rates of transfusions in laparoscopic resections when compared to open resections (10% *vs* 48%).

Operative time: In terms of Operative time, the average operative time ranged from 95 to 280 min. Although one study showed increased operative time when compared to open surgery[33], 3 studies demonstrated decreased operative time in laparoscopic surgery[5,24,48]. Dagher *et al*[47] showed decreased operative times when early experience is compared to an established technique period. (270 ± 143 min *vs* 171 ± 95 min, $P < 0.001$), which points out that there is a learning curve with lapa-

Table 2 Reported survival after laparoscopic resection for hepatocellular carcinoma

Ref.	Disease-free survival				Overall survival			
	1 yr	2 yr	3 yr	5 yr	1 yr	2 yr	3 yr	5 yr
Gigot *et al*[59]		43.70%				62.50%		
Chen *et al*[53]					85.4%-94.7%		66.4%-74.2%	59.4%-61.7%
Tranchart *et al*[36]			60.90%	45.60%			74.40%	59.50%
Yoon *et al*[8]			60.40%				90.40%	
Lai *et al*[30]	85%		47%	38%	96%		67%	52%
Belli *et al*[31]	81%		62%	32%	95%		70%	55%
Truant *et al*[32]				35.50%				70%
Lee *et al*[33]	78.80%		51%	45.30%	86.90%		81.80%	76%
Hu *et al*[34]								50%
Yoon *et al*[25]			52.90%	46.40%			93.30%	85.90%
Choi *et al*[23]			71%				81%	
Inagaki *et al*[45]								79.30%

roscopic liver surgery.

Conversion to open surgery and length of stay: Conversion to laparotomy rates in the literature ranged from 0% to 19.4%. Koffron *et al*[48] converted to Hand-assisted technique in 6% of the resections and had 0% conversion to open surgery which signifies that the use of the hand-assisted technique may decrease the rate of conversion to open surgery. Ikeda *et al*[27] had a 0% conversion rate in a series of 30 patients. In the series published after 2011, conversion rates never exceeded 6%, which also may point to a learning curve effect in laparoscopic liver surgery. The main reasons for conversion were bleeding, adhesions, and inability to complete the resection laparoscopically due to technical or anatomical considerations.

The average length of stay ranged from 4.8 to 13 d in the studies and many of them found a shorter length of stay when compared to open surgery[4-6,26,33,53,58].

Morbidity and mortality: The average morbidity rate was 5.5% to 27.5%. Common complications included bile leak, liver abscess, and transient hepatic failure. General complications included pleural effusions, pneumonia, urinary tract infection, and cardiac arrhythmia. Lower morbidity as compared to open surgery was observed in many studies[5,6,24,26,48].

Inagaki *et al*[38] showed markedly decreased rate of pulmonary complications in the patients who underwent laparoscopic resection as compared to open surgery (3% *vs* 17%).

Mortality rate varied between 0% and 3.7%. Reported causes of death included liver failure[28,58], cerebral infarction secondary to hypotension[9], postoperative hepatorenal failure[17], pseudomembranous colitis, technical clip failure leading to massive hemorrhage[14], acute respiratory distress syndrome[28,31], bleeding from esophageal varices[13], and multi-organ failure[35].

Potential disadvantages: While the safety of laparoscopic liver resection has been documented, possible limitations include the significant learning curve[47], loss of tactile feedback and inability to manually palpate the liver that may cause missing other lesions, and potential bleeding that may be harder to control laparoscopically[2].

Oncologic results

Resection margin: The surgical margins were free of cancer in 87% to 100% of patients in the published studies. The Size of the margin varied with an average ranging between 0.3 cm to 2.08 cm. Topal *et al*[6] showed a lower rate of positive surgical margin with laparoscopic surgery as opposed to open resection; 1.2% *vs* 2.1%.

Castaing also showed higher rate of R0 resection with laparoscopic resection 87% compared to open surgery 72%[14]. The addition of intraoperative ultrasound was associated with lower rate of positive margins[59]. Buell *et al*[17] reported the only case of port site metastasis in the literature which was in a patient who underwent laparoscopic resection for CRM.

Hepatocellular carcinoma: Hepatocellular carcinoma is the most common primary liver malignancy. 52% of reported laparoscopic liver resections done for malignancy are performed for HCC[2]. In the literature, the 3-year disease-free survival was 51% to 62% and the overall 3-year survival ranged from 66.4% to 90.4%. The 5-year disease-free survival ranged from 32% to 46.4%, while 5-year overall survival was 50% to 85.9% (Table 2).

Three studies found no difference in survival between patients with hepatocellular carcinoma who were treated with laparoscopic resection when compared to open resection[32,33,52].

Belli *et al*[60] showed reduced morbidity, especially postoperative ascites, and a shorter length of stay in cirrhotic patients who underwent laparoscopic resection for hepatocellular carcinoma when compared to those who underwent open resection.

In the largest series of 163 patients with hepatocellular carcinoma treated at 3 European centers by Dagher *et al*[28], the mean operative time was 180 min, mean estimated blood loss was 250 mL, the mortality rate was 1.2% and the mean resection margin was 1.2 cm. The researchers also found that there has been a decrease in operative time and blood loss, increase in the size of tumors re-

Table 3 Reported survival after laparoscopic resection for colorectal metastasis

Ref.	Disease-free survival				Overall survival			
	1 yr	2 yr	3 yr	5 yr	1 yr	2 yr	3 yr	5 yr
Gigot *et al*[59]		53.3%				100%		
O'Rourke *et al*[7]	81%				88%			
Castaing *et al*[14]	65%		30%	30%	97%		82%	64%
Shafaee *et al*[29]			27%				83%	55%
Lai *et al*[30]	92%		72%		100%		88%	
Topal *et al*[24]	61.3%	41.8%		29.9%	94.5%	83.5%		59.5%
Nguyen *et al*[62]	65%		43%	43%	88%		69%	50%

sected, and lower rate of morbidity in patients who were operated on in the later experience series[28].

Colorectal liver metastasis

Surgery is the only curative option for metastatic colorectal cancer to the liver. 35% of laparoscopic liver resections for malignancy are performed for CRM[2].

The goal of resection in patients with CRM is resection of all liver metastasis with a negative margin but at the same time preserving adequate post-resection liver volume. Liver resection offers long-term survival in up to 60% of patients with CRM[61].

Disease-free survival at 1 year was 61.3% to 81%, but dropped at 5 years to 30% to 42%. Overall survival at 1 year was 88% to 100%, at 3 years ranged from 82% to 88%, and at 5 years ranged from 51% to 64% (Table 3).

Nguyen *et al*[62] reported the largest series of 109 patients with laparoscopic resection for CRM. The mean estimated blood loss was 200 mL, the transfusion rate was 10%, the average length of operation was 234 min, the resection margin was negative in 94.4%, and the conversion rate was 3.7%. Conversion was due to hemorrhage.

Shafaee *et al*[29] showed that the survival rates for patients undergoing repeat hepatectomy laparoscopically were equivalent or even superior to the long-term outcomes of patients undergoing open resection. Two other studies have showed no statistical difference in disease-free and overall survival rates between laparoscopic and open surgery[14,24].

CONCLUSION

In conclusion, Although laparoscopic liver surgery requires a great deal of experience by hepatobiliary surgeons, this approach is deemed safe for malignant liver lesions with comparable oncological results to open surgery. In addition, it offers better short term results including shorter hospital stay, lower costs, less postoperative pain, and lower complication rates. While operative blood loss has been a major concern with laparoscopic resection, a number of reports is showing that minimally invasive approach is associated with lower risk of bleeding and transfusions. Tumor size is no longer an obstacle to laparoscopic resection, although laparoscopic major hepatectomy is still limited to experienced centers. The disadvantages of the laparoscopic approach are the loss of tactile sensation and palpation of the liver as well as the learning curve that is associated with laparoscopic resection. It should be noted that there has been no randomized clinical trials involving laparoscopic liver resection for malignancy and such trial is needed to clarify the risks *vs* benefits and help standardize the approach.

COMMENTS

Background

After being initially described for small peripheral benign tumors, laparoscopic liver resection for malignant and larger lesions is now reported extensively in the literature.

Research frontiers

This purpose of this study is to review the published literature on laparoscopic liver resection for malignant lesions. Authors discuss the different type of resection, the most common as well as the innovative techniques, the surgical outcomes, postoperative complications, and oncologic results.

Innovations and breakthroughs

Although laparoscopic liver surgery requires a great deal of experience by hepatobiliary surgeons, this approach is deemed safe for malignant liver lesions with comparable oncological results to open surgery.

Peer review

This manuscript thoroughly reviews the history and advances on the laparoscopic resection for liver malignancy, including the indications and contraindications, minor and major resections, hand-assisted technique, robotic resection, operative technical details, and the outcomes. The text is generally well written.

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CASE REPORT

Problem of living liver donation in the absence of deceased liver transplantation program: Mansoura experience

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Abstract

We report our experience with potential donors for living donor liver transplantation (LDLT), which is the first report from an area where there is no legalized deceased donation program. This is a single center retrospective analysis of potential living donors (n = 1004) between May 2004 and December 2012. This report focuses on the analysis of causes, duration, cost, and various implications of donor exclusion (n = 792). Most of the transplant candidates (82.3%) had an experience with more than one excluded donor (median = 3). Some recipients travelled abroad for a deceased donor transplant (n = 12) and some died before finding a suitable donor (n = 14). The evaluation of an excluded donor is a time-consuming process (median = 3 d, range 1 d to 47 d). It is also a costly process with a median cost of approximately 70 USD (range 35 USD to 885 USD). From these results, living donor exclusion has negative implications on the patients and transplant program with ethical dilemmas and an economic impact. Many strategies are adopted by other centers to expand the donor pool; however, they are not all applicable in our locality. We conclude that an active legalized deceased donor transplantation program is necessary to overcome the shortage of available liver grafts in Egypt.

Key words: Living donor; Liver transplantation; Excluded donors; Deceased donor; Liver disease

Core tip: This is the first case series from a country where a deceased donor liver transplantation program is not available and the shortage of living liver donors is high. We report our experience regarding the problem of excluded donors and possible strategies to overcome this problem. We hope that this experience will be of benefit to the readers of the World Journal of Gastroenterology.

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INTRODUCTION

Each center may have its own unique criteria for potential donors of living donor liver transplantation (LDLT) regarding the geographical and cultural backgrounds, however, each center must follow the current global standards of care[1-4]. In Egypt, a deceased donor liver transplantation (DDLT) program is still awaited, thus, LDLT is the only hope for patients with end-stage liver disease or hepatocellular carcinoma in the presence of cirrhosis[5]. Our experience regarding the short- and long-term impact of liver transplantation on living donors has been published in a previous study[5].

Few studies have examined the reasons for excluding living liver donors and its impact on transplantation. These studies have been conducted in countries where DDLT is allowed and the problem of donation is not so high. Most available articles are based on a small number of potential donors and some of these articles included donors who were excluded for reasons related to the recipient[6-9].

In this study, we investigated the impact of excluding living donors on patients, the transplant team and medical resources in the absence of a DDLT program. We discuss different possible strategies to overcome this problem and the expected outcome following the adoption of these strategies in our center.

CASE REPORT

Study subjects

This is a retrospective cohort study of excluded donors (n = 792) from potential donors (n = 1004) for LDLT using a right lobe graft during the period from May 2004 to December 2012 in the Liver Transplant Program, Gastrointestinal Surgical Center, Mansoura University, Egypt. Potential donors were any living donors who came to our center after the recipient had been informed of the accepted donor criteria. Excluded donors were those who were not permitted to undergo living liver donation to a certain recipient due to reasons related to the donor, irrespective if the decision was made by the transplant team, recipient, or the donor himself.

Data collection

Data for this study were retrieved from the internal web-based registry system supplemented by paper-based records of the potential donors included in the medical files of the corresponding recipients. Data were collected and rearranged in a standardized manner including details on donor age, sex, body mass index (BMI), liver function tests, viral serological markers, results of liver biopsy and imaging studies if performed, cause and phase of exclusion, duration from the donor's first visit to the exclusion decision, and the cost of any investigations and special consultations carried out.

Donor evaluation

Donor candidates were thoroughly evaluated according to the multidisciplinary protocol shown in Table 1. A multistep consent process involving two different surgeons and a hepatologist on separate occasions was used, during which the operative procedure and potential complications were described in detail. Donors were informed that the risks of morbidity and mortality were 40% and 0.5%, respectively[5].

Non-related living donation was accepted only when the patient was approved by an independent ethical and legal committee and none of his relatives were suitable right liver lobe donors. The legal age of consent for donation in Egypt is 18 years when the recipient is a parent, otherwise it is 21 years. The upper age limit for living liver donation is 45 years. According to the 2006 Census held by the Central Agency for Mobilization and Statistics (CAPMAS), only 6.27% of Egyptians are aged above 60 years[10]. This implies that most chronic diseases occur at a younger age in Egyptians in comparison to other populations. This justifies our choice of an upper age limit of 45 years, although many centers in other countries accept higher age limits, even greater than 60 years.

Routine liver biopsy in the living liver donor evaluation protocol is controversial. Some centers have adopted routine liver biopsy after unfortunate experiences with living donors including operation abortion and donor mortality due to undiscovered congenital lipodystrophy[6,11]. For safety, liver biopsy in living liver donors is necessary. Thus, liver biopsy is routinely performed by an experienced radiologist.

Liver biopsy is performed prior to imaging studies such as hepatic angiography, magnetic resonance cholangiopancreatography (MRCP) and liver volumetry to evaluate the anatomy of the vascular and biliary systems. Although the former is an invasive procedure, it is far less expensive (50 USD) than the imaging studies (500 USD). Also, liver biopsy had been performed in our center since 1974 to evaluate patients with portal hypertension for shunt operations and with this cumulative experience the procedure is almost risk-free. The burden of the donor evaluation costs is on the recipient with little support from the state. Therefore, we perform liver biopsy prior to imaging studies for economic reasons, especially as liver biopsy is almost risk-free in our center.

Donors with a BMI > 35 were re-evaluated after they committed to a successful weight loss program. Absolute exclusion criteria were ABO incompatibility, pregnancy, mental inadequacy, an underlying medical condition that was considered to increase the risk of complications, positive hepatitis B or C serology, underlying liver disease, steatosis > 30%, and abnormal anatomy considered by surgeons to increase the risk of hepatectomy or affect the remaining liver.

Statistical analysis

The Shapiro-Wilk test was used to assess normality of the data. Numerical data are presented as means and standard deviations or as medians with ranges. A P value < 0.05 was considered statistically significant. Statistical analysis was carried out using IBM SPSS v. 20.

Table 1 Living donor evaluation protocol

Potential donor: Is any living donor at our center after the recipient is informed of the accepted donor criteria. The criteria for living liver donors include an apparently healthy relative of the recipient aged between 21 and 45 years. Recipient, donor and their families are informed regarding the risks and benefits of right lobe hepatectomy for LDLT and both the donor and the recipient must sign an informed written consent to proceed to the evaluation process
Phase Ⅰ: Confirmation of the relation between the living donor and the candidate recipient by the independent legal and ethical committee, confirmation of being within the accepted age limits, assessment of BMI, initial anesthetic and surgical evaluation, confirmation of ABO compatibility, complete blood count (CBC) and biochemistry assays, pregnancy test, abdominal Doppler ultrasound, serological tests for hepatitis C and B viruses (serum HCV RNA qualitative and quantitative assay using PCR, HBV surface antigen, HB core antibody, HB e antigen, HB e antibody, HB surface antibody), serological assessment for different viruses (human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), Herpes virus Ⅰ and Ⅱ, Cytomegalovirus (CMV), Chagas disease and syphilis), electrocardiography (ECG), echocardiography, plain X-ray of the chest, exclusion of specific liver diseases (ferritin, transferrin, and serum iron), and reassessment after previous investigations
Phase Ⅱ: Liver biopsy for assessment of degree of steatosis and detection of any pathological conditions
Phase Ⅲ: Hepatic angiography to delineate the anatomy of the portal vein, hepatic artery and hepatic veins. Magnetic resonance cholangiopancreatography (MRCP) is performed to assess the anatomy of the biliary system. Liver volumetry to assess the right lobe graft size, residual donor liver volume and graft-recipient weight ratio (GRWR). The donor is accepted if GRWR > 0.8 and the residual donor liver volume ⩾ 30%
Phase Ⅳ: All donors must sign a written informed consent including agreement to participate in LDLT

LDLT: Living donor liver transplantation.

Table 2 Number and ratio for each reason of donor exclusion during phase Ⅰ of the living donor evaluation protocol

Phase	Step	*n*	Exclusion	*n*	% from the step
Phase Ⅰ	Confirmation of the relative	1004	Non-related donor	2	0.19
	Age	1002	Over age limit	223	22.25
	BMI	779	Overweight	26	3.33
			Underweight	5	0.64
	Clinical evaluation	748	Uncontrolled HTN	2	0.26
	Initial surgical evaluation	746	Upper abdominal surgery	6	0.8
	Psychological evaluation	740	Algophobia	1	0.13
			Hesitation	3	0.4
	ABO compatibility	736	ABO incompatibility	102	13.85
		634	Withdrawal	23	3.62
	CBC and biochemistry	611	thrombocytopenia	3	0.49
			uncontrolled DM	4	0.65
			elevated liver enzymes	7	1.14
			hyperbilirubinemia	2	0.32
			hypoalbuminemia	1	0.16
		594	Family refusal	5	0.84
			Withdrawal	6	1.01
	Pregnancy test	583	Positive test	16	2.74
	Abdominal US	567	Splenomegaly	1	0.17
			Fatty liver	1	0.17
		565	Withdrawal	29	5.13
	Serological assessment	536	HCV positive	39	7.27
			HBV positive	34	6.34
			CMV positive	7	1.3
			EBV positive	1	0.18
			HCV and HBV positive	6	1.11
			HCV and CMV positive	1	0.18
		448	Withdrawal	9	2.0
	Anesthetic reassessment	439	Smoker	28	6.37
			IHD	2	0.45
			Rheumatic heart disease	3	0.68
			Valvular heart disease	3	0.68
			Atrial ectopics	1	0.22
		402	Withdrawal	31	7.71

HCV: Hepatitis C virus; HBV: Hepatitis B virus; CMV: Cytomegalovirus.

Reasons for exclusion

Between May 2004 and December 2012, we received 1004 potential donors in the Liver Transplant Program, Mansoura Gastrointestinal Surgical Center. Two hundred and twelve donors (21.1%) met the inclusion criteria for transplant surgery, while 792 candidates (78.8%) were excluded. Most of these donors were excluded during phase Ⅰ (Table 2) of the evaluation protocol (*n* = 633, 79.9%), while exclusion in other phases was as follows (Table 3): phase Ⅱ (*n* = 108, 13.6%), phase Ⅲ (*n*

Table 3 Number and ratio for each reason of donor exclusion during phase Ⅱ to Ⅳ of the living donor evaluation protocol

Phase	Step	*n*	Exclusion	*n*	% from the step
Phase Ⅱ	Liver biopsy	371	Moderate steatosis	16	4.31
			Severe steatosis	9	2.42
			Portal tract fibrosis	27	7.27
			Bilharzial granuloma	10	2.69
			Reactive hepatitis change	4	1.07
			Focal portal infiltrate	1	0.26
			Portal lymphocyte infiltrate	1	0.26
			Hemosiderosis	1	0.26
			Focal necrotic areas	1	0.26
		301	Withdrawal	38	12.62
Phase Ⅲ	Volumetry and anatomical assessment	263	Portal venous variants	2	0.76
			Biliary variants	6	2.28
			hepatic venous variants	8	3.04
			hepatic venous and biliary variants	1	0.38
			hepatic and portal venous variants	3	1.14
			Small for size graft	15	5.70
			Small residual left lobe	4	1.50
		224	Withdrawal	9	4.01
Phase Ⅳ	Informed consent	215	Withdrawal	3	1.39
	Donors underwent surgery	212			

= 48, 6.1%) and phase Ⅳ (*n* = 3, 0.4%).

The most frequent reason for donor exclusion was exceeding the upper age limit (*n* = 172, 21.7%), while 6.4% of potential donors (*n* = 51) were below the lower age limit. ABO incompatibility represented the 2nd most common cause of exclusion during phase I and the 3rd most common cause in the whole evaluation process with a ratio of 12.9% (*n* = 102). 2% of potential donors (*n* = 16) were found to be pregnant. Twenty-six (3.3%) candidate donors were excluded due to BMI > 30, and 5 donors (0.6%) were excluded because they were underweight. One donor was excluded because he had algophobia. Two potential donors were excluded as they were unrelated to the patient, which was discovered during confirmation by the independent ethical and legal committee.

One hundred and forty-eight donors (18.7%) withdrew during the evaluation process. Most of them withdrew during the first phase (*n* = 98), while 38 potential donors withdrew during the second phase and 9 withdrew during the third phase. Three donors withdrew after completion of the evaluation process on the night of surgery. Three candidate donors expressed much hesitation during first interviews with the transplant team and were excluded. Interestingly, 5 donors were excluded as their families refused donation. Of these 5 cases with family refusal, one father refused his daughter's donation to himself and a husband refused his wife's donation to her mother. Of the potential donors, 6 donors were excluded because they had previous nephrectomy and two of them frankly stated they wanted a renal transplant.

In a country where hepatitis C virus (HCV) is endemic, 39 potential donors (4.9%) were excluded because they were serologically positive for HCV infection. Thirty-four (3.4%) were serologically positive for HBV, 6 candidates (0.8%) were positive for both HCV and hepatitis B virus (HBV), 7 candidates (0.9%) were positive for cytomegalovirus (CMV), one candidate was positive for both HCV and CMV and one candidate was serologically positive for Epstein-Barr virus (EBV). Ten candidates had abnormal liver function tests in the form of elevated liver transaminase enzymes (*n* = 7), elevated serum bilirubin (*n* = 2) and hypoalbuminemia with hypoprothrombinemia (*n* = 1). One donor was excluded because he had fatty liver on ultrasound. One of the excluded donors had splenomegaly and 3 candidates were excluded because they had thrombocytopenia.

The anesthesia team excluded 28 candidates (3.5%) because they were smokers who refused to stop smoking before surgery. They excluded other potential donors because they had uncontrolled diabetes mellitus (*n* = 4), uncontrolled hypertension (*n* = 2), ischemic heart disease (*n* = 2), rheumatic heart disease (*n* = 3), valvular heart disease (*n* = 3), and atrial ectopic activity (*n* = 1).

Twenty-five candidates (3.2%) were excluded because they had moderate to severe macrovesicular steatosis on liver biopsy. Many donors were also excluded during assessment in phase Ⅱ following liver biopsy which revealed portal tract fibrosis (*n* = 27, 3.4%), active bilharzial granuloma (*n* = 10, 1.3%), reactive hepatitis changes (*n* = 4, 0.5%), focal portal infiltrate and interface hepatitis (*n* = 1, 0.1%), portal lymphocytic infiltrate (*n* = 1, 0.1%), mild hemosiderosis (*n* = 1, 0.1%) and hepatic focal necrotic areas (*n* = 1, 0.1%).

During phase Ⅲ of evaluation, 15 candidates (1.9%) were excluded because liver volumetry revealed decreased right lobe size suspected to cause small for size graft, while 4 donors had a small left lobe which would result in inadequate reserve in the donor after surgery. Anatomical assessment by angiography and MRCP excluded many candidates because of unsuitable hepatic venous variants (*n* = 9), unsuitable biliary variants (*n* = 6), unsuitable portal venous variant (*n* = 1), unsuitable hepatic and portal venous variants (*n* = 3) and unsuitable hepatic venous and biliary variants (*n* = 1).

Impact on the recipients

Of 212 patients who underwent LDLT, 204 patients (96.2%) had an experience with an excluded donor with a median number of 3 donors per recipient (range 1 to 56 excluded donors). Eight patients (3.7%) did not experience donor exclusion and they underwent liver transplantation. Fourteen patients did not have a suitable living related donor and died due to end-stage liver disease. Twelve patients travelled abroad and underwent surgery in countries where LDLT is legalized, particularly China (n = 7).

Impact on the transplant team

The decision to withdraw was made by the donor in 148 cases and by pressure from family in 5 cases. The exclusion decision was made by the transplant team in 639 cases. The median duration between the candidate's first visit to our center and the exclusion or withdrawal date was 3 d ranging from 1 d (n = 151) to 47 d (n = 1).

Economic impact

The average cost of the investigations carried out for donor assessment in phase Ⅰ, phase Ⅱ and phase Ⅲ was 150 USD, 200 USD and 700 USD, respectively. 1004 candidates entered phase Ⅰ and 371 completed phase Ⅱ. 301 candidates completed phase Ⅱ and 224 potential donors completed phase Ⅲ. Of those who completed phase Ⅲ, 9 candidates withdrew and 215 completed Phase Ⅳ, after which 3 patients withdrew and 212 underwent transplant surgery. The median cost of the investigations performed for the excluded donor evaluations was 70 USD ranging from 35 USD (n = 103) to 885 LE Should this be USD? (n = 3). 264 donors (33.3%) were excluded without any need for special investigation. The burden of the expenses for donor evaluation lies with the recipient.

DISCUSSION

There is a worldwide paucity of liver donors in countries where deceased, living and domino liver transplants are all available, while in Egypt there is the problem of endemic hepatitis C liver cirrhosis with only living donors available. Through our experience with 1004 potential living donors, we encountered a lot of problems that involved the patients and transplant team; some of these problems had social roots with ethical and economic implications. This study describes the importance of a DLT program even after adoption of various strategies to expand the living donor pool, although we believe that it should not be adopted until after public debate and agreement.

A number of studies have detailed the evaluation protocol for living liver donors. Although donor exclusion is discussed in the context of reporting the experience with LDLT[11,12], to our knowledge, very few reports have exclusively discussed the impact of donor exclusion[6-9]. Apart from differences in the evaluation protocols between these reports, some authors excluded donors due to reasons related to the recipient in their study. This absence of uniform criteria for the presentation of data makes it difficult to compare our study with previous studies. We believe that this is the first study of a large number of potential donors from an area where there is no DLT program.

The evaluation of living donors for LDLT is a stepwise process consisting of a comprehensive medical work-up, psychological assessment and accurate anatomical delineation. Donor evaluation is an expensive labor-intensive and time-consuming process. The evaluation process should start with non-invasive steps, which are less expensive and detect a greater number of unsuitable donors and then progress to more invasive and expensive tests[6]. Based on our above-mentioned evaluation protocol, 26.62% of potential donors were excluded before undergoing any investigations.

In our series, 6.9% of potential donors were excluded based on abnormal liver biopsy mainly due to steatosis (2.48%) or bilharzial liver insult in the form of active granuloma or portal tract fibrosis (3.67%). This ratio is lower than the ratio reported in other studies by Valentín-Gamazo *et al*[6] (8%), Pomfret *et al*[13] (22%) and Marcos[14] (17%).

Donor withdrawal during, or even after, evaluation for LDLT is an inevitable cause of donor exclusion. An ethical dilemma occurs when a competent donor candidate gives voluntary informed consent, but his family refuses his donation. Although we adopt patient-centered Western bioethics, undue pressure from the candidate's family puts the team in a difficult situation. How can you proceed to surgery when the family calls the police to the hospital alleging that the donor is coerced? Should you not consider a husband who threatens to divorce his wife if she donates her right liver lobe to her mother? These dilemmas are not well discussed in the literature, although there is a significant focus on donors under pressure from the family to donate to a relative, not the reverse!

In our experience, 22.21% of potential donors were excluded because their age was outside the accepted age range in our center, which is between 21 and 45 years old. The accepted age for living liver donation varies between centers. The lower age limit is determined by the law which defines the accepted age of consent[6,15]. In Egypt, the legal age of consent is 18 years old when the recipient is a parent, otherwise it is 21 years old. The upper age limit is variable and most centers exceed 50 years old and may even be up to 75 years old. Other centers consider old age a risk factor that if associated with other risk factors, such as nicotine abuse or hypertension, the donor is excluded, otherwise he is accepted[6,11,15,16]. In comparison to these populations, the National Census revealed that only about 6% of Egyptians are aged above 60 years and that is why we chose an upper age limit of 45 years old[10].

In this series, 10.15% of potential donors were excluded because of ABO incompatibility. Possible solutions include ABO incompatible LDLT and paired donor exchange. The main concern in ABO incompatible transplantation is antibody-mediated rejection (AMR). Prophy-

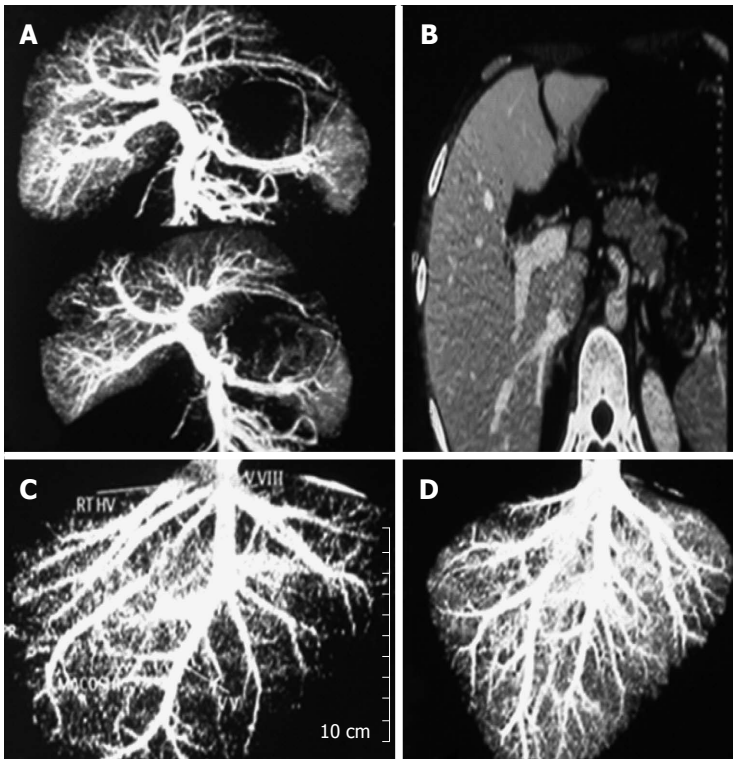

Figure 1 Examples of excluded donors due to unsuitable anatomical variations as shown by imaging studies. (A) Computerized tomographic (CT) portography showing the right portal vein arises from the left portal vein (B) CT volumetry showing that drainage of right lobe is mainly through the middle hepatic vein (C) CT venography showing drainage of the right lobe through multiple hepatic veins (D) CT venography showing small right hepatic vein and inferior right hepatic vein draining into the middle hepatic vein.

laxis against AMR includes antiCD20 antibody (rituximab), splenectomy, reinforced immunosuppression and local infusion of prostaglandin E1 (PgE1) and/or steroids[17-19]. ABO incompatible transplantation is less favorable than compatible transplantation due to inferior long-term patient survival[19-21]. Exchange donor transplant was first adopted in Korea in 2003. The two main principles of the procedure are equality and simultaneity meaning that the two pairs are operated on at the same time. The bad news is that the rate of paired donor exchange after long-term application in Korea did not exceed 10%[15].

Of 536 candidates who were serologically assessed for hepatitis B and C markers in our center, 14.9% of them were accidentally discovered to be serologically positive for HCV and/or HBV. Twenty-three potential donors were excluded because they were hepatitis B core antibody (HBcAb) positive and hepatitis B surface antigen (HBsAg) negative. This ratio was lower than those reported in endemic regions where it reached up to around 50% in potential donors[22]. There is a risk of *de novo* hepatitis B infection in naïve recipients for HBV who receive grafts from HBcAb positive donors[22,23]. Immunoprophylaxis in naïve recipients using hepatitis B immunoglobulin (HBIG), lamivudine and vaccination is associated with a lower incidence of *de novo* infection[24]. However, the low prevalence of HBV infection together with the expensive postoperative prophylactic course (11700 USD), which the recipient cannot afford, has led to refusal of the use of donors with positive HBcAB.

Precise and accurate knowledge of the anatomy of hepatic vasculature and the biliary system is of paramount importance in planning the best resection and to lessen the risk of morbidity. With refinement of surgical techniques and cumulative experience, exclusion of potential donors due to anatomical variations has decreased over time, however, there are still conditions where donor safety necessitates "no go" hepatectomy even after starting the operation[25]. In our experience, 3.38% of potential donors were excluded on an anatomical and volumetric basis. Examples of cases excluded due to anatomical and volumetric considerations are shown in Figure 1.

Due to legislative obstacles supported by social background, deceased donor liver transplantation is not yet available in Egypt. The paucity of suitable willing living donors and an absence of grafts from deceased donors have resulted in many Egyptian patients seeking transplants abroad. In nearly all cases, the follow-up and management of post-transplant complications is carried out in Egypt. Thus, this so called "transplant tourism" has a great impact on the progress of the LDLT program in Egypt[26]. Significant effort is needed to establish a comprehensive legal system allowing deceased and living donor liver transplantation with airtight safeguards against donor coercion, commercialism and time-consuming meaningless obstacles[27].

In conclusion, dependence on LDLT is insufficient, especially in a country where HCV-induced liver cirrhosis is prevalent. Many strategies can be adopted to increase the living liver donor pool. Most, if not all, of these strategies have risks for the donor (older donors) or for the recipient (ABO incompatible LDLT and HBcAb positive donors). Legalization of DLT is essential to cope with the increasing number of patients in need of liver transplantation.

COMMENTS

Case characteristics

In this study, the authors investigated the impact of excluded living donors on patients, the transplant team and medical resources in Egypt which does not have a deceased donor liver transplantation program.

Experiences and lessons

Apart from differences in the evaluation protocols between reports, some authors also excluded donors due to reasons related to the recipient in their study. The absence of uniform criteria for the presentation of data makes it difficult to compare our study with previous studies. The authors believe that this is the first study of a large number of potential donors from an area where there is no DLT program.

Peer review

Authors reported a retrospective cohort study of excluded donors from potential living-donor for liver transplantation, using the right lobe graft during 8.5 years in Egypt. They suggested that living donor liver transplantation is not enough especially in a country where hepatitis C virus induced liver cirrhosis is prevalent, and that strategies to increase the donor pool may involve a risk on the marginal donor or on the recipient with ABO incompatibility and/or with HBcAb(+) donors. They also concluded that legalization of deceased donor for liver transplantation is required. A death concept is so different between countries, and we have respect for if when a deceased-donor will be legislated.

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CASE REPORT

Meckel's diverticulum incarcerated in a transmesocolic internal hernia

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Abstract

Intestinal obstruction is a common complication associated with Meckel's diverticulum in adults. The diverticulum itself or its fibrous band can lead to an intestinal volvulus, intussusceptions, or closed-loop obstructions, which require surgery. The incarceration of Meckel's diverticulum in either inguinal or femoral hernia sacs (Littre's hernia) is another, less common, etiology underlying intestinal obstruction. This case report describes a 45-year-old man who had an obstruction associated with a Meckel's diverticulum that passed through a congenital defect in the mesocolon into the right subphrenic space. The patient, who had not undergone abdominal surgery previously, came to the emergency room with acute onset of intermittent epigastric pain and abdominal distention. Computed tomography images showed the presence of a segment of the small bowel and a diverticulum in the right subphrenic space and paracolic gutter. The twisted mesentery and the dilated loops of the proximal small bowel were indicative of an intestinal volvulus and obstruction. Meckel's diverticulum complicated by a transmesocolic internal hernia was diagnosed, and this condition was confirmed during emergency surgery. The patient's postoperative recovery was uneventful. This case report highlights another presentation of Meckel's diverticulum, that is, in combination with a transmesocolic internal hernia. This etiology may lead to an intestinal volvulus and necessitate early surgery.

Key words: Meckel's diverticulum; Intestinal obstruction; Hernia; Abdominal

Core tip: Intestinal obstruction is a common complication associated with Meckel's diverticulum in adults. It can also be complicated with transmesocolic internal hernia, which had not been reported before. Early surgery is necessary since it could result in intestinal volvulus and closed-loop obstruction. High clinical suspicion in acute abdomen is important while computed tomography scans helps preoperative diagnosis.

Wu SY, Ho MH, Hsu SD. Meckel's diverticulum incarcerated in a transmesocolic internal hernia. *World J Gastroenterol* 2014; 20(37): 13615-13619 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13615.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13615

INTRODUCTION

Obstruction of the small bowel originating from congenital abnormalities is uncommon among adults. The correct diagnosis of obstructions of the small bowel requires differentiation of normal anatomy from pathologic findings using radiography, and the presence of a high index of clinical suspicion. Meckel's diverticulum is the most common congenital anomaly of the gastrointestinal tract. Adult patients affected by Meckel's diverticulum may be asymptomatic until they experience acute small-bowel obstruction, which may be caused by either the fi-

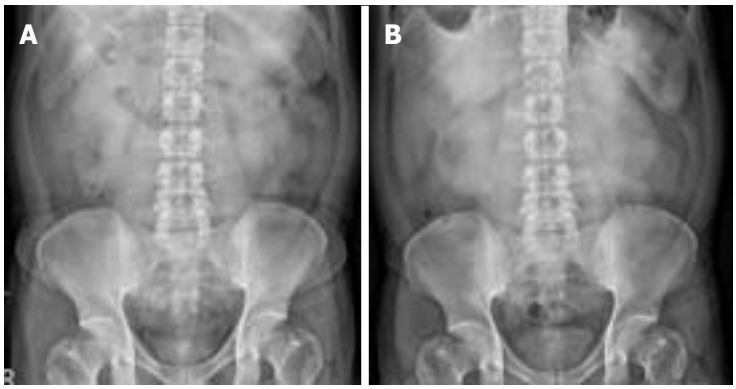

Figure 1 Serial abdominal radiographs showing gas within the small bowel overlying the liver and relative radiolucencies lateral to the liver, suggesting intestinal incarceration. The intestinal diverticulum between the diaphragm and liver is prominent in the area of the latter. A: 5 mo ago; B: During the acute episode.

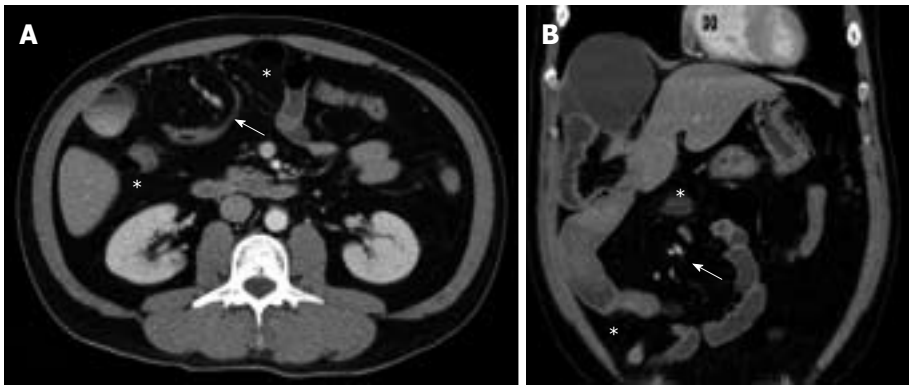

Figure 2 Computed tomography scans showing the incarceration of Meckel's diverticulum and the small bowel in the right subphrenic space, causing the depression of liver surface. The twisted mesenteric vessels (arrow) are indicative of a volvulus; the group of small-bowel loops lateral to the colon (asterisk) also suggest a transmesocolic internal hernia. A: Axial view; B: Coronal view.

brous band of the diverticulum or a volvulus of the small bowel around the diverticulum[1]. However, incarceration of Meckel's diverticulum within a transmesocolic internal hernia is an unusual etiology. Here, we describe the case of a patient who had an acute obstruction of the small bowel. An incarcerated Meckel's diverticulum in the right subphrenic space with an intestinal volvulus was found on computed tomography (CT) images. In addition, a transmesocolic internal hernia was found during radiography, and this finding was confirmed during surgery.

CASE REPORT

A 45-year-old man who did not have a history of systemic diseases and had no history of abdominal surgery was admitted to our emergency room (ER). On the day before he came to the ER, he had experienced acute onset of intermittent epigastric pain and abdominal distention, with nausea, vomiting, and diarrhea. Several years before being admitted to the ER, the patient had experienced episodes of dull epigastric pain, accompanied by bloating and constipation. However, the patient had ignored the pain because the intensity of the pain fluctuated and the pain appeared to be self-limiting. Initial physical examinations showed increased level of bowel sounds, no abdominal tenderness, and no palpable masses. Results from the patient's blood tests were normal. Plain-film radiographic images of the abdomen did not show the ileus, but revealed relative radiolucencies lateral to the liver. Compared with a plain film obtained in the outpatient department 5 mo previously, these images showed more prominent presence of bowel gas between the liver and diaphragm (Figure 1). Eight hours after being admitted to the ER, the patient's colic became increasingly severe and more frequent, and diffuse abdominal tenderness was apparent on physical examination. Emergency CT scans showed incarceration of one segment of the intestine in the right subphrenic space where the mesenteric vessels and the soft tissues twisted around each other in the prehepatic space, which is known as a "whirl sign" (Figure 2). Furthermore, a segment of the strangulated intestine or a diverticulum of the intestine depressed the liver surface and displaced the hepatic flexure of the colon medially. However, free air and ascites were not present within the subphrenic space. We suspected a volvulus and bowel ischemia, and we performed a diagnostic laparoscopy, which was converted to a laparotomy after confirmation of the ischemic bowel. A 20-cm segment of the ileum passed through a 10-cm defect in the transverse mesocolon into the subphrenic space, 30 cm proximal to the ileocecal valve. Within this, a 6-cm antimesenteric diverticulum was trapped in the subphrenic space (Figure 3). The diverticulum served as the lead point of mesenteric twisting, causing bowel ischemia. After clamping the mesenteric vessels, segmental resection and end-to-end anastomosis of the ileum were performed. The histopa-

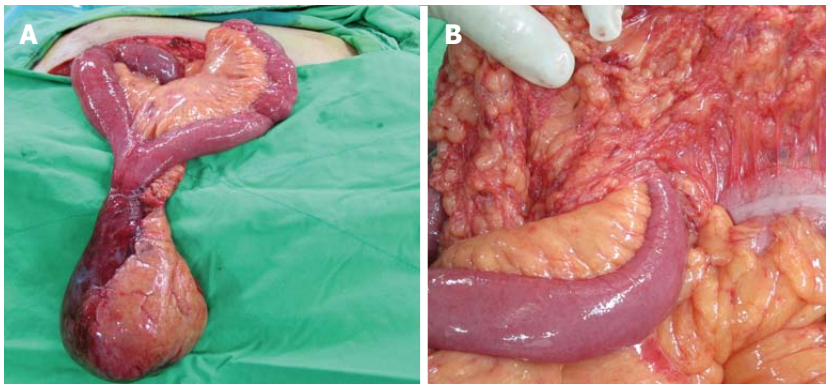

Figure 3 Intraoperative photographs. A: The herniated Meckel's diverticulum and small bowel, showing the completion of the manual reduction of the mesenteric volvulus; B: The inlet of the internal hernia in the transverse mesocolon.

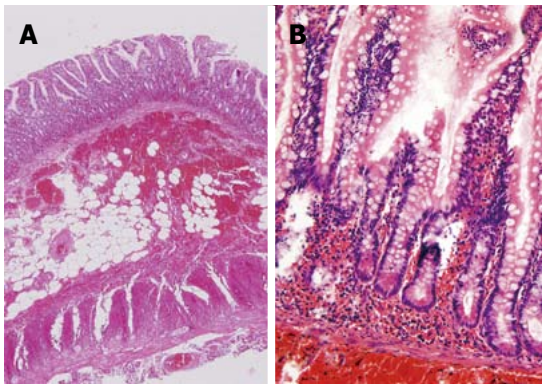

Figure 4 Hematoxylin eosin stains of the resected diverticulum showing mucosal infarction, extensive submucosal hemorrhage, and inflammatory cell infiltration of the tissues of the ileum and continuous muscle layer, suggesting acute bowel ischemia. A: × 25; B: ×200.

thology of the diverticulum showed mucosal infarction and excessive submucosal hemorrhage, with neutrophil infiltration in the ileum tissue. The bowel wall contained all layers without metaplasia of gastric or pancreatic mucosa (Figure 4). These findings were more suggestive of bowel ischemia rather than acute diverticulitis since no dense lymphocyte infiltration was observed in the lamina propria. These findings are compatible with those of a true congenital diverticulum, namely, Meckel's diverticulum. The patient recovered well after surgery. He was discharged from hospital, without complications, after 9 d.

DISCUSSION

Meckel's diverticulum, a true diverticulum arising from the antimesenteric side, is the most common congenital anomaly of the gastrointestinal tract. While most patients with Meckel's diverticula do not experience complications, between 4% and 16% of patients experience complications[1]. Bleeding in association with ectopic gastric mucosa is the most common complication in children, whereas obstructions caused by intussusceptions or adhesion bands are more common in adults. Furthermore, a Meckel's diverticulum may protrude through an abdominal opening. In Littre's hernia, a Meckel's diverticulum can enter the inguinal canal, umbilical canal, or femoral canal, causing incarceration or strangulation[2]. Sometimes, the adhesion band of the diverticulum forms a space that may cause the internal herniation of the small intestine[3]. However, the protrusion of a Meckel's diverticulum through a congenital defect in the mesocolon is extremely rare. Indeed, when we searched the medical literature published in English, we could not find a case that was similar to the one that is reported here.

During the development of internal abdominal hernias, the intestines intrude into a cavity or defect in the peritoneal cavity. Internal hernias comprise up to 5.8% of intestinal obstructions[4]. Most internal hernias involve intestines passing through physiologic openings, and they may be categorized as paraduodenal hernias, pericecal hernias, foramen of Winslow's hernias, or inter-sigmoid hernias. Other internal hernias are associated with congenital or acquired defects, and they are categorized as transmesenteric, transmesocolic hernias, and transomental hernias[5,6]. Without intestinal strangulation, patients may have no symptoms or insidious, vague, postprandial abdominal pains, which are often ignored. Most patients are not given a diagnosis until the nausea, vomiting, and the acute, cramping, periumbilical pain, become suggestive of an intestinal obstruction, and CT images are critical in these diagnoses. The location of the herniated loops of the small bowel is anatomically abnormal, and they may displace adjacent organs and cause the engorgement, crowding, twisting, or stretching of the mesenteric vessels[7]. The location of the loops of the small bowel helps determine etiology. For left paraduodenal hernias, the small bowel enters a congenital fossa behind the ascending duodenum, and bowel loops are present between the stomach and pancreas. In contrast, in right paraduodenal hernias, bowel loops are present just lateral and inferior to the descending duodenum. In paracecal hernias, the bowel loops are located posterior and lateral to the cecum in the right paracolic gutter. In intersigmoid hernias, the incarcerated small bowel is posterior and lateral to the sigmoid colon. In transmesenteric hernia, the small bowel travels through a mesenteric defect to the lateral side of the colon and displaces the overlying omentum, creating a directly abutting abdominal wall. Most transmesenteric

hernias in adults are acquired and are secondary to previous abdominal surgery such as Roux-en-Y anastomoses for gastric bypass or hepaticojejunostomies. In contrast, congenital defects are more frequent in children. Since our patient had none of the etiologies that would have led to the acquisition of an internal hernia, including previous abdominal surgery, trauma, or peritonitis, the defect in the mesocolon was probably congenital. CT scans showed the entrapped intestinal diverticulum in the right subphrenic space depressing the liver's surface. Furthermore, the small intestine was located lateral to the ascending colon and displaced the hepatic flexure of the colon downwards, indicating a possible internal hernia. While an intestinal volvulus was also suggested by the twisted mesenteric vessels and the proximal dilation of the incarcerated small intestine, the incarcerated small intestine and the diverticulum may have also served as a leading point. The emergency surgery warranted by this closed-loop intestinal obstruction confirmed that the Meckel's diverticulum passed through a congenital defect in the mesocolon and into the right subphrenic space with an intestinal volvulus.

As the images illustrate, the intestines were in the right subphrenic space, and in these instances Chilaiditi's sign, an indication of colonic interposition between the liver and the diaphragm, should always be considered. Chilaiditi's sign is usually an incidental finding on abdominal radiographs and it tends to exist without any symptoms. Relaxation of the suspensory ligaments mobilizes the right colic flexure, making interposition possible. When this is accompanied by symptoms associated with bowel obstruction, including abdominal pain, nausea, vomiting, bloating, and anorexia, it is called Chilaiditi's syndrome[8]. However, a diagnosis of Chilaiditi's syndrome should be differentiated from diseases associated with acute abdomen, which include pneumoperitoneum, subphrenic abscess, obstructions of the small bowel, volvulus, intussusceptions, ischemic bowel, and inflammatory conditions, for example, appendicitis or diverticulitis, all of which require prompt surgical intervention. In contrast, Chilaiditi's syndrome is managed by medical treatment initially, rather than surgery. Surgical intervention, in the form of cecopexy or colonic resection, is indicated when medical treatment fails, or if there is evidence of bowel ischemia, for example, volvulus of the transverse colon or cecum. Our patient had an acute abdomen, and CT scans showed a small-bowel volvulus and a closed-loop obstruction. The normal proximal ascending colon, cecum, and terminal ileum excluded the colonic obstruction that characterizes Chilaiditi's syndrome. Our patient's radiologic findings were confirmed by diagnostic laparoscopy.

A radiologic finding of a small bowel trapped between the diaphragm and the liver requires careful evaluation. A patient who presents with an acute abdomen requires emergency diagnostic laparoscopy or laparotomy. Furthermore, it is important to exclude internal hernias by identifying the relative anatomic locations of the intra-abdominal organs on CT images in patients who have incidental findings. If congenital anomalies such as Meckel's diverticulum are apparent on CT images, we suggest prompt surgical investigation, because of risk of an intestinal volvulus.

COMMENTS

Case characteristics

A 45-year-old man with acute intermittent epigastric pain, abdominal distention, nausea, vomiting, and diarrhea.

Clinical diagnosis

increased level of bowel sounds, no abdominal tenderness initially then progression to diffuse tenderness , no palpable masses.

Differential diagnosis

Intestinal obstruction related to congenital anomaly or neoplasm, Chilaiditi syndrome.

Laboratory diagnosis

White blood cell: 13560/uL; C-reactive protein: 0.14 mg/dL; metabolic panel and liver function test were within normal limits.

Imaging diagnosis

CT scans showed incarceration of one segment of the intestine in the right subphrenic space where the mesenteric vessels and the soft tissues twisted around each other in the prehepatic space.

Pathological diagnosis

Meckel's diverticulum with ischemic change secondary to volvulus.

Treatment

Laparotomy with reduction of transmesenteric internal hernia and resection of incarcerated ileum including Meckel's diverticulum.

Related reports

Others reported the adhesion band of the diverticulum forms a space that may cause the internal herniation of the small intestine. But we could not find a case like ours with Meckel's diverticulum passing through a congenital defect in the mesocolon.

Experiences and lessons

In patient with clinical diagnosis of intestinal obstruction, internal hernias are differentiated by identifying the relative anatomic locations of the intra-abdominal organs on CT scans. Combination of Meckel's diverticulum and transmesenteric internal hernia is possible, and we suggest prompt surgical investigation even in asymptomatic patients because of risk of an intestinal volvulus.

Peer review

This is a well written case report of surgical interest. On histology the authors should comment on the presence of acute inflammation indicating if there was also acute diverticulitis as now they only describe absence of metaplasia.

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CASE REPORT

Active gastrointestinal diverticulum bleeding diagnosed by computed tomography angiography

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Author contributions: Xu XQ and Liu W designed the report; Xu XQ, Hong T, Li BL and Liu W were the attending doctors for these patients; Xu XQ, Hong T and Li BL performed the surgical operations; Xu XQ and Liu W organized the report; Xu XQ wrote paper.
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Abstract

A diverticulum is a bulging sack in any portion of the gastrointestinal tract. Small intestine diverticular disease is much less common than colonic diverticular disease. The most common symptoms include non-specific epigastric pain and a bloating sensation. Major complications include diverticulitis, gastrointestinal bleeding, acute perforation, intestinal obstruction, intestinal perforation, localized abscess, malabsorption, anemia, volvulus and bacterial overgrowth. We report one case of massive jejunal diverticula bleeding and one case of massive colonic diverticula bleeding, both diagnosed by acute abdominal computed tomography angiography and treated successfully by surgery.

Key words: Diverticulum; Gastrointestinal diverticular bleeding; Angiography; Computed tomography angiography; Endoscopy

Core tip: Gastrointestinal diverticular disease is common and asymptomatic in most cases; however, massive gastrointestinal bleeding caused by diverticular disease is rare. This article describes the diagnosis and management of these diseases through acute abdominal computed tomography angiography.

Xu XQ, Hong T, Li BL, Liu W. Active gastrointestinal diverticulum bleeding diagnosed by computed tomography angiography. *World J Gastroenterol* 2014; 20(37): 13620-13624 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13620.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13620

INTRODUCTION

A diverticulum is a bulging sack in any part of the gastrointestinal tract. The large intestine is the most common site for the formation of diverticula. The diverticulum forms a pouch in a weakness of the intestine, which generally corresponds to the site where an artery enters into the muscle layer. It is thought that spasms increase pressure in the intestine, creating more diverticula and enlarging existing ones. Diverticula are classified as real or false: real diverticula are composed of all of the intestine's layers, while false diverticula consist of the uncus of the mucosa and the submucosa[1].

Diverticular disease is actually uncommon in people under the age of 40. By the age of 50, about 1/3 of the population has diverticulosis; the prevalence increases to 60% in people over 80 years of age[2].

Colonic diverticular disease is much more common than small intestine diverticular disease. It is believed that diverticula develop as a result of abnormalities in intestinal peristalsis, intestinal dyskinesis and very high intraluminal pressure, such as in the colon[3].

Most gastrointestinal diverticula are asymptomatic;

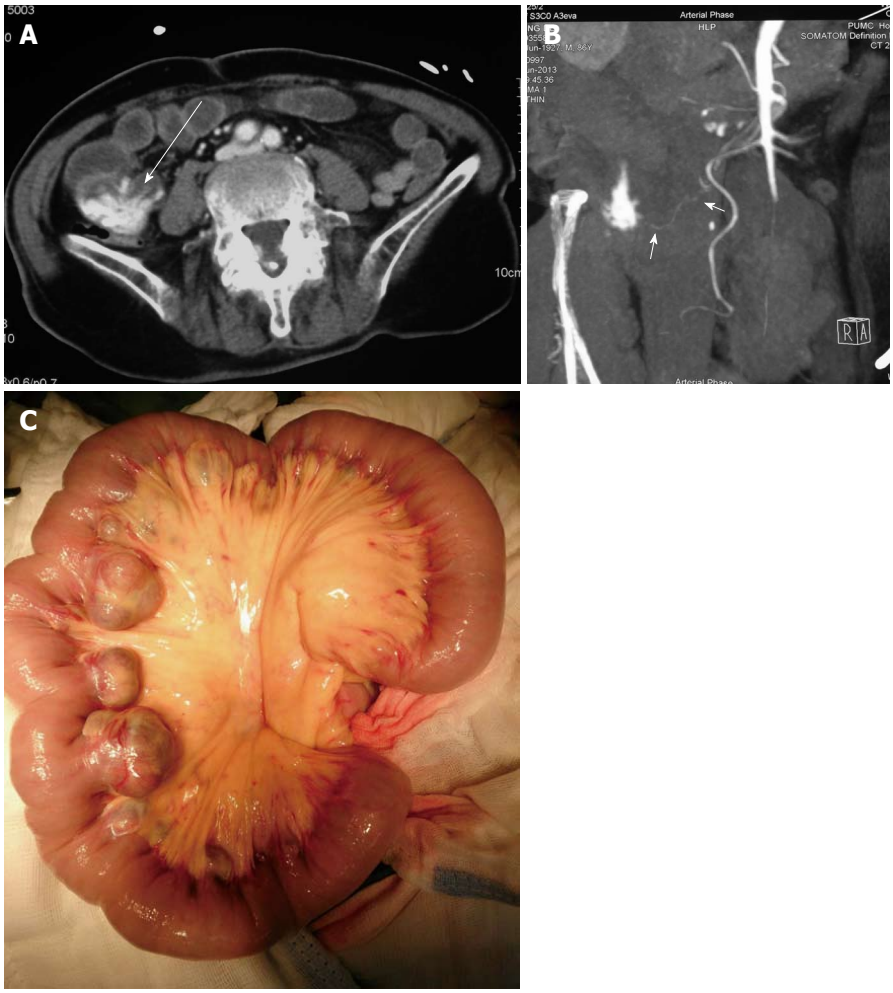

Figure 1 Imaging of case 1. A: Computed tomography (CT) angiography revealed contrast extravasation in a jejunal diverticulum (arrow); B: CT angiography showed the blood supply for the jejunal diverticulum source was from a branch of the superior mesenteric artery (arrow); C: Multiple diverticula in a 30 cm segment of the jejunum.

however, they are usually complicated by diverticulitis with or without bowel perforation, intestinal obstruction and bleeding once they become symptomatic. Massive upper gastrointestinal bleeding secondary to gastrointestinal diverticulosis is extremely rare. Most of them present with obscure gastrointestinal bleeding[4-6]. However, colonic diverticular bleeding is a common cause of hematochezia. It accounts for approximately 40% of the episodes of massive lower gastrointestinal bleeding and often results in hospitalization. Severe hemorrhage can occur in 3%-5% of patients with diverticula[7]. Diverticula are predominantly observed in the left colon in Western countries, while right-sided diverticula are more commonly found in Asian countries[8].

Computed tomography (CT) angiography, colonoscopy and angiography are essential for the diagnosis and treatment of gastrointestinal diverticular bleeding[1-8]. However, a precise location and definite hemostasis are often difficult because diverticula are usually numerous and bleeding is intermittent[6-8]. Blind segmental resection in the cases of undiagnosed bleeding site is responsible for the high re-bleeding rate of 42% and mortality of 57%[9]. Therefore, precise localization is essential for the treatment in patients with a life-threatening diverticular bleeding. We herein describe two patients with massive jejunal and colonic diverticular bleeding, respectively, whose bleeding sites were located precisely and successfully by CT angiography and then treated by surgery.

CASE REPORT

Case 1

An 86-year-old male presented to the emergency department with recurrent massive hematochezia. His past medical history was significant for hypertension for 20 years without any history of gastrointestinal bleeding. On physical examination, the abdomen was soft without tenderness. His hemodynamic status was unstable, with a hemoglobin of 6.5 g/dL. An acute abdominal CT angiography revealed contrast extravasation in a jejunal diverticulum (Figure 1A, arrow). The superior mesenteric artery was reconstructed, which indicated the blood supply for the jejunal diverticulum source was from a branch of the superior mesenteric artery (Figure 1B, arrow). Once the diagnosis of jejunal diverticular bleeding was made, a laparotomy was performed. Multiple diverticula

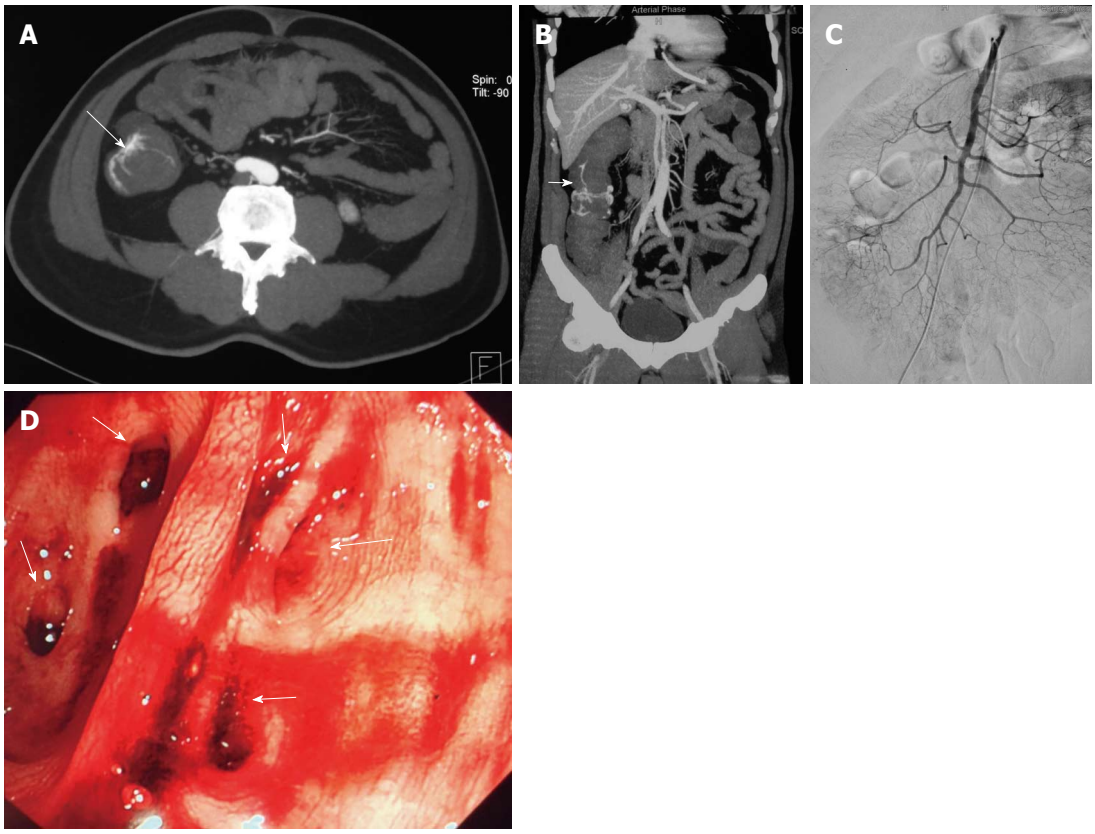

Figure 2 Imaging of case 2. A: Abdominal computed tomography (CT) revealed contrast extravasation in the ascending colon (arrow); B: CT angiography revealed bleeding from the intramural branches of the marginal artery supplying the ascending colon (arrow); C: Angiography failed to reveal the precise location of the bleeding; D: Emergent colonoscopy revealed multiple diverticula in the middle part of the ascending colon without definite bleeding vessels identified (arrow).

in a 30 cm segment of jejunum were found, beginning 50 cm distal to the Treitz's ligament (Figure 1C). The involved segment was resected. The patient had no further postoperative episodes of gastrointestinal bleeding.

Case 2

A 56-year-old male presented to the emergency with recurrent massive hematochezia. His past medical history was non-significant, with no history of gastrointestinal bleeding. On physical examination, the abdomen was soft without tenderness. His hemodynamic status was unstable (BP 85/50 mmHg, HR 110/min) with a hemoglobin of 5.4 g/dL. The acute abdominal CT angiography revealed contrast extravasation in the ascending colon (Figure 2A, arrow) and bleeding from the intramural branches of the marginal artery supplying the ascending colon (Figure 2B). Angiography failed to reveal the precise location of the bleeding (Figure 2C). Emergent colonoscopy was performed, which revealed multiple diverticula in the middle part of ascending colon (Figure 2D, arrows), but no definite bleeding vessels were identified. When the conservative treatment for colonic diverticular hemorrhage was unsuccessful after three episodes of hemorrhage, a laparotomy was performed. Partial colectomy for most of the ascending colon was then performed. The patient had no further post-operative episodes of gastrointestinal bleeding.

DISCUSSION

Gastrointestinal diverticular disease is asymptomatic in up to 70% of cases and patients may suffer from acute complications such as bleeding, perforation, diverticulitis or intestinal obstruction[4-6]. Bleeding is uncommon and constitutes only 5%-33% of all cases[10]. Both upper and lower gastrointestinal diverticular usually involve elderly patients[2]. A significant number of patients also present with life-threatening bleeding. Based on these facts, clinicians should keep in mind with this uncommon but dangerous situation while managing elderly patients with massive gastrointestinal bleeding.

Preoperative diagnosis of gastrointestinal diverticular bleeding is sometimes difficult[11,12]. Many modalities have been introduced to evaluate gastrointestinal diverticular bleeding, including barium contrast study, Technetium^{99m} (^{99m}Tc) RBC scan, CT scan, angiography, CT angiography, capsule endoscopy, *etc*.

The upper gastrointestinal diverticular bleeding is much less common than the lower gastrointestinal diverticular bleeding (colonic diverticular disease). The duodenum is the second most common location for gastrointestinal tract diverticula after the colon. Other less common locations include the third and fourth portions of the duodenum and the jejunum[13].

Colonic diverticula are the most common source of

life-threatening lower gastrointestinal bleeding. Most diverticula are located in the descending or sigmoid colon; however, spontaneous bleeding is common in diverticula of the ascending colon[14].

Emergent endoscopy is the standard diagnostic and therapeutic technique for gastrointestinal bleeding, but it can be difficult when the typical gastrointestinal bleeding involves fast and massive bleeding into an unprepared bowel. As a matter of fact, no exact explanation is found in up to 20% in such cases of gastrointestinal bleeding. Arteriography is reserved for patients for whom endoscopy is unfeasible or inconclusive, and for whom bleeding persists after localization; however, it risks ischemic damage to the bowel wall and failure to detect all bleeding sources. CT angiography can be useful in locating the obscure source of bleeding. CT angiography showed pooled sensitivity of 86% and specificity of 95%, respectively in the diagnosis of acute gastrointestinal bleeding[15]. When massive gastrointestinal bleeding occurs in patients with unstable hemodynamic status, CT angiography is essential, it could show sac-like collections of extravasation, so that details of its arterial supply can be clearly ascertained in order to optimize treatment decisions[16].

CT angiography is accurate in the diagnosis of acute gastrointestinal bleeding and can show the precise location and etiology of bleeding. Thus, CT angiography could be used for the first line diagnostic method for most of the patients with active gastrointestinal bleeding without relative absolute contraindications[15].

The management of gastrointestinal diverticular bleeding is mainly by surgical intervention, although there are some reports of spontaneous recovery. The mortality rate has dropped in recent years. The decrease in mortality may be attributed to the advance of diagnostic modalities that allow clinicians to stratify patients for endoscopic therapy, conservative treatment, or surgery[17]. For those with less severe bleeding, endoscopic diagnosis and treatment are good options[18]. A significant number of patients are expected to bleed again during the follow-up period, although such a non-surgical approach does control the bleeding. For those presented with massive bleeding, emergency surgery still remains one of the options to control the bleeding. Whether these patients should receive surgery after initial medical management remains unclear. This approach may be suggested only when they suffer from recurrent bleeding. Surgery is indicated in patients with ongoing recurrent bleeding when therapeutic endoscopy or angiography is not available or feasible. Patients with recurrent gastrointestinal bleeding should certainly be treated with surgery because the risk of further episodes may increase with time[19]. Approximately 10%-25% of patients require emergency surgery for hemodynamic instability[20].

In conclusion, CT angiography is a noninvasive, rapid, reproducible and widely available technique that can be performed successfully upon the majority of patients presenting with an acute episode of gastrointestinal diverticula bleeding. Positive contrast-enhanced multidetector CT can define the bleeding sites with high accuracy. Occasionally, this technique can even define the cause of active gastrointestinal bleeding, which, if combined with anatomical information, can effectively inform subsequent angiography or surgery. Multiphasic imaging enables direct visualization of active bleeding into the bowel. CT angiography could serve as a first diagnostic modality in the evaluation of patients with substantial bleeding[21].

COMMENTS

Case characteristics

Active gastrointestinal diverticulum bleeding diagnosed by computed tomography (CT) angiography.

Differential diagnosis

It should be considered in the differential diagnosis of patients with gastrointestinal bleeding caused by any other reasons.

Diagnostic imaging

Diagnostic imaging mainly by CT angiography.

Treatment

Angiography or surgery may be performed when definite bleeding sites are identified by CT angiography.

Peer review

This is a well written and well-documented report of two cases of active gastrointestinal diverticular bleeding.

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CASE REPORT

Intra-abdominal inflammatory myofibroblastic tumor: Spontaneous regression

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Abstract

Inflammatory myofibroblastic tumors are usually treated by surgical resection. We herein report two cases of intra-abdominal inflammatory myofibroblastic tumors that were unresectable and underwent spontaneous regression without any treatment. Our case report and literature review show that regression is more common in the middle-aged and older male populations. Abdominal discomfort and fever were the most common symptoms, but the majority of patients had no obvious physical signs. There was no specific indicator for diagnosis. The majority of the lesions regressed within 3 mo and nearly all of the masses completely resolved within 1 year. We conclude that the clinical characteristics of inflammatory myofibroblastic tumors are variable and, accordingly, the disease needs to be subdivided and treated on an individual basis. Surgery is always the first-line treatment; however, for those masses assessed as unresectable, conservative therapy with intense follow-up should be considered.

Key words: Inflammatory myofibroblastic tumor; Inflammatory pseudotumor; Abdominal cavity; Spontaneous regression

Core tip: This article reports two rare cases involving the spontaneous regression of an unresectable intra-abdominal inflammatory myofibroblastic tumor (IMT) and summarizes the clinical characteristics of all published cases. The clinical characteristics of IMT are not clear and the treatment is controversial. By analyzing published papers, we discovered some common features of the disease and potential reasons for this unusual phenomenon. Our findings may be useful for the clinical diagnosis and treatment of IMT.

Zhao JJ, Ling JQ, Fang Y, Gao XD, Shu P, Shen KT, Qin J, Sun YH, Qin XY. Intra-abdominal inflammatory myofibroblastic tumor: Spontaneous regression. *World J Gastroenterol* 2014; 20(37): 13625-13631 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13625.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13625

INTRODUCTION

Inflammatory myofibroblastic tumor (IMT), also called inflammatory pseudotumor (IPT), is a rare disease commonly found in children and adolescents[1].

The etiology of IMT remains unclear, but is most likely associated with inflammation, trauma[2], viral infection[3], chromosome translocation[4], and gene fusion[5]. Although IMT has long been profiled as a benign lesion, it has presented aggressive behavior in some cases due to local spread, recurrence, and metastasis[6,7]. In 2003, IMT

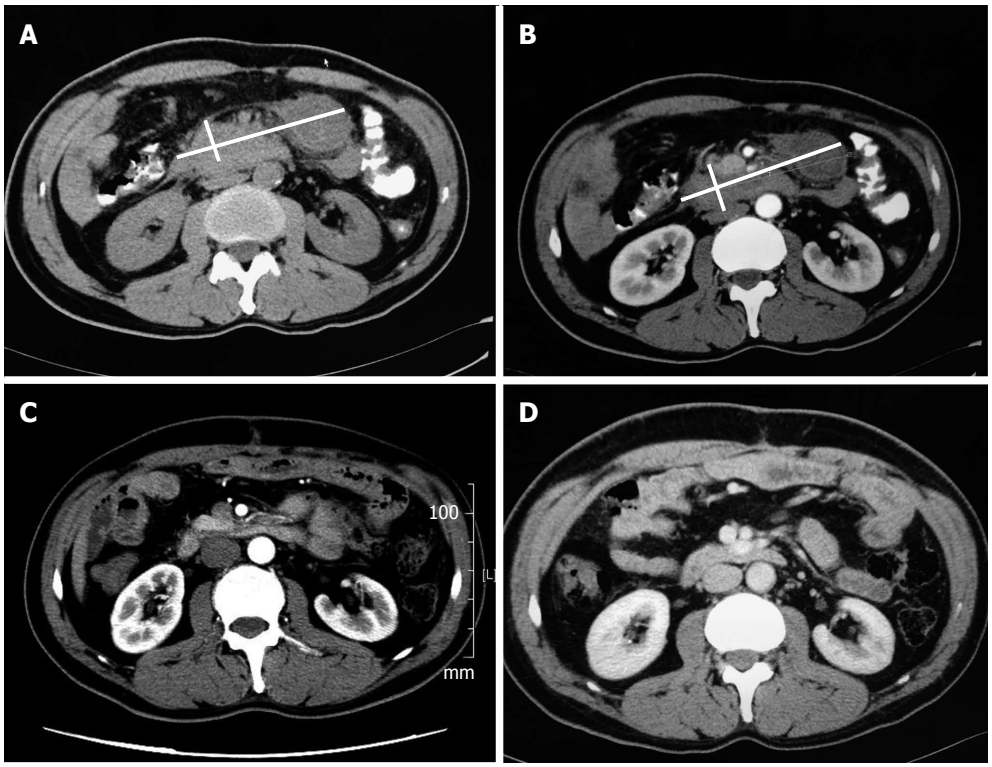

Figure 1 Computed tomography imaging changes of patient No. 1 A: A plain computed tomography (CT) scan on admission showing a large nodular mass (white lines mark the maximum and minimum diameter) located in the upper quadrant of the peritoneum and encasing the surrounding soft tissues; B: An enhancement CT scan showing the mass to be multinodular. Some parts of the mass were mildly enhanced, while others were cystic degenerated; C: A CT scan obtained 3 wk after the operation identified the spontaneous regression of the tumor; D: A CT scan obtained 3 mo later did not show any indication of relapse.

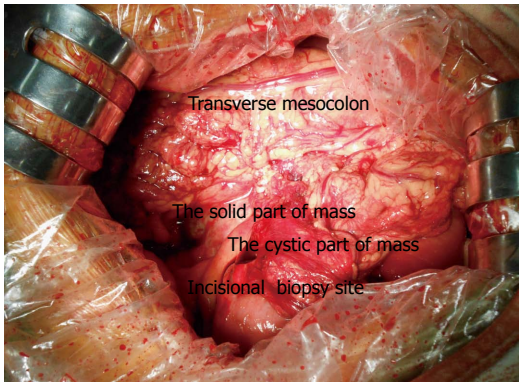

Figure 2 Intraoperative photograph of patient No. 1. A solid-cystic mass was discovered invading adjacent organs and tissues during exploration. The mass was approximately 15 cm × 8 cm in size and could not be entirely removed.

was classified as an intermediate neoplasm in the current World Health Organization (WHO) histologic typing[7] and characterized by a mixture of myofibroblasts, fibroblasts, lymphocytes, and plasma cells[8].

Surgery has long been regarded as the most effective and radical treatment. However, there were sporadic cases which reported the effectiveness of using antibiotics[9], corticosteroids[10], nonsteroidal anti-inflammatory drugs (NSAIDs)[2], and even non-intervention[11], which may provide new insights into the therapeutic approach for IMT, especially for unresectable lesions.

Herein, we present two cases of unresectable IMT arising from the abdominal cavity, both eventually resolving completely without any treatment. We also reviewed the relevant literature, with an emphasis on the spontaneous regression of intra-abdominal IMT, and summarized the clinical characteristics of these patients.

CASE REPORT

Case 1

A 49-year-old male sought medical treatment with symptoms including paroxysmal abdominal pain, nausea, and vomiting, which recurred over 2 wk. A large mass was palpated in the epigastrium. Laboratory tests showed elevated neutrophil count (6.7 × 10^9/L), C-reactive protein (CRP) (27.9 mg/L), and lactic dehydrogenase (383 U/L). Tumor markers were normal except for CA125 (44.8 IU/mL). A computed tomography (CT) scan of the abdomen revealed the presence of a very large nodular mass in the upper quadrant of the peritoneum (Figure 1A and B). Although the diagnosis was not confirmed, the clinical evidence indicated that the lesions were likely to be a malignant tumor. Based on this diagnosis, the patient was admitted to surgery. During exploration, a 15 cm × 8 cm large mass was discovered in the retro-peritoneum that was attached to the posterior wall of the abdomen (Figure 2). We performed an incisional biopsy of the mass at different sites. The histological diagnosis was of a fibro-inflammatory proliferation, with immunohistochemistry staining results showing negative expressions of CD34,

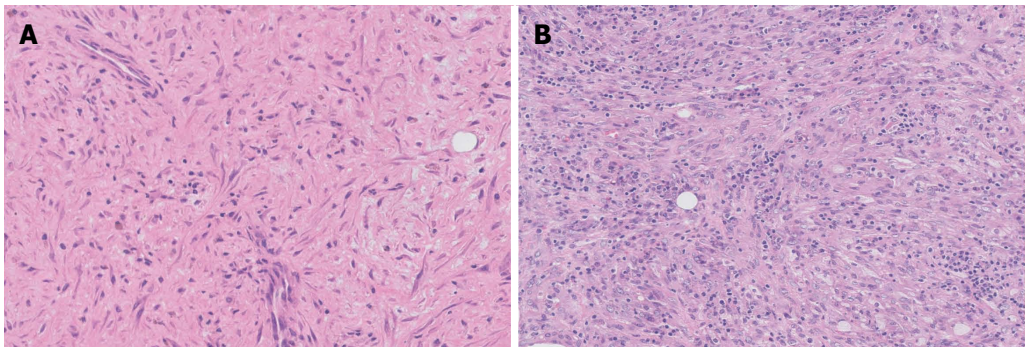

Figure 3 Pathological images of two patients. A: Histological examination showing a proliferation of myofibroblasts and infiltration of inflammation cells (HE stain, 200 ×); B: Histological examination showing a proliferation of spindled cells, likely myofibroblasts, mixed with abundant lymphocytes and plasma cells (HE stain, 200 ×).

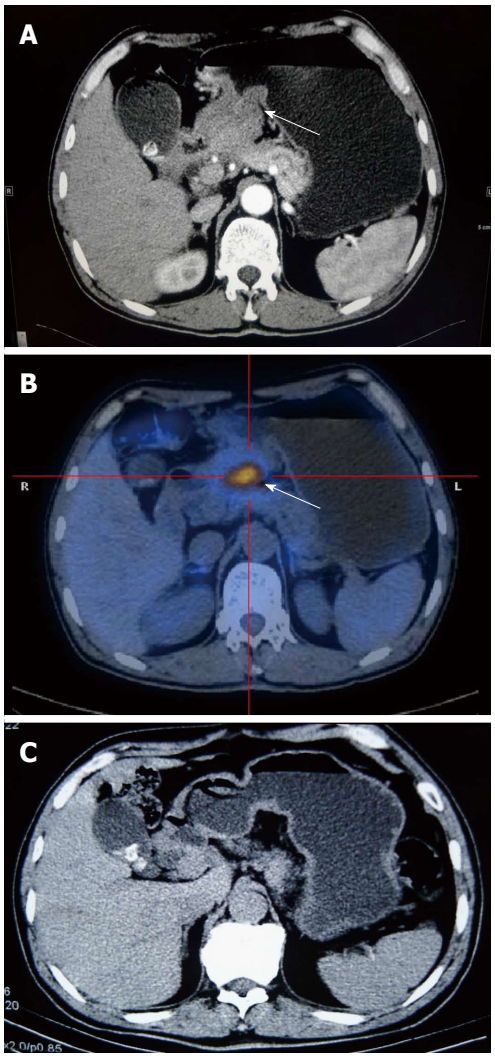

Figure 4 Computed tomography/positron emission tomography-computer tomography imaging changes of patient No. 2. A: A computed tomography (CT) scan on admission showed that the gastric wall at the antrum was clearly thickened and enhanced (white arrow) in the arterial phase. Several lymph nodes could also be observed, the biggest one being approximately 3.9 cm in diameter; B: A positron emission tomography-computer tomography on admission showed a high FDG (fluorodeoxyglucose) uptake mass located at the interval between the antrum and pancreas (white arrow). The maximum standardized uptake value (SUV max) was 11.7, and the retention index was 21.8%; C: A CT scan obtained 1 mo after discharge identified the spontaneous regression of the tumor.

desmin, and anaplastic lymphoma kinase (ALK), as well as a positive expression of SMA, which is consistent with IMT (Figure 3A). Because the mass was unresectable, we only performed gastrojejunostomy to relieve the obstruction. After surgery, the patient presented with delayed gastric empting and was completely symptom-free 3 wk later. Before discharging, the patient was re-examined by CT scan, which showed the apparent and spontaneous regression of the mass without a residual small tumor or inflammation remaining (Figure 1C). A CT scan three months later showed no indication of relapse (Figure 1D).

Case 2

A 59-year-old man was hospitalized with a 1-mo history of abdominal distension, poor appetite, and weight loss of more than 5 kg. He was addicted to alcohol and tobacco and had uncontrolled diabetes mellitus. Clinical examination was unremarkable other than mild abdominal tenderness. Laboratory tests, including a routine hepatic function test, showed that C-reactive protein and tumor markers were all within the normal range. Gastroscopy detected an elevated lesion at the pylorus, which was likely from the extra-cavity. We twice performed an endoscopic biopsy, but both findings were of superficial gastritis. A CT scan and a PET-CT scan both demonstrated that a presumptive malignant mass was located between the gastric antrum and the neck of the pancreas (Figure 4A and B). Although we did not acquire a pathological diagnosis, we performed an exploratory laparotomy. During the surgery, we confirmed that the mass was located at the posterior wall of the gastric antrum and had invaded the pancreas, transverse mesocolon, and mesentery, which resulted in an inability to perform an en bloc resection. We only removed the subpyloric lymph nodes for a pathological assay. Histological examination showed conspicuous proliferation of myofibroblasts admixed with abundant inflammatory cells (Figure 3B), and the immunohistochemistry results demonstrated that CD34, desmin, and ALK were negative, while SMA was positive. Based on these findings, the diagnosis of IMT was made. The patient did not receive any treatment and became symptom-free one month later. The tumor mass regression was verified using a CT scan (Figure 4C). After

Table 1 Published reports about spontaneous regression of intra-abdominal Inflammatory myofibroblastic tumor

Ref.	Year	Cases *n*	Location
Gollapudi *et al*[12]	1992	1	Liver
Jaïs *et al*[13]	1995	1	Liver
Su *et al*[2]	2000	1	Mesentery
Koea *et al*[10]	2003	6	Liver
Biecker *et al*[14]	2003	1	Liver
Thompson *et al*[15]	2003	1	Mesentery
Tanikawa *et al*[16]	2003	1	Periureteral
Przkora *et al*[17]	2004	2	Retro-peritoneum
Colakoglu *et al*[18]	2005	1	Liver
Koide *et al*[19]	2006	1	Liver
Tsou *et al*[9]	2007	4	Liver
Yamaguchi *et al*[20]	2007	3	Liver
Vassiliadis *et al*[21]	2007	1	Liver
Motojuku *et al*[22]	2008	1	Liver
Mattei *et al*[8]	2008	1	Retro-peritoneum
Goldsmith *et al*[23]	2009	5	Liver
Brage-Varela *et al*[24]	2010	1	Liver
Jerraya *et al*[11]	2011	1	Liver
Fragoso *et al*[7]	2011	2	Liver and pancreas
Shatzel *et al*[1]	2012	1	Mesentery

discharging, the patient underwent a CT scan every three months at a local hospital and did not have any indication of recurrence during a one-year follow-up.

Literature review

A systematic search of PubMed and Embase was performed. We listed 20 reports published from 1992 to date in Table 1, with a sum of 36 intra-abdominal IMT or IPT patients who had spontaneous regression without surgical excision[1,2,7-24]. In an effort to assess the biological behavior of IMT, we summarized the clinical characteristics of a total of 38 patients consisting of 36 reported cases as well as our two cases.

Age and gender: Including our two cases, the median age of patients was 44 years (ranging from a few months to 79 years). The largest population was the middle-aged and elderly, with a percentage of 60.6%. There were 25 male patients, nearly twice as many as female patients.

Symptoms: Twenty-five of 31 patients were symptomatic at presentation (no data were available for 7 cases). The most common symptoms were abdominal pain (58.1%), fever (45.2%), weight loss (22.6%), appetite loss (12.9%), and nausea/vomiting (12.9%). Nearly 19.4% of patients were completely asymptomatic.

Physical examination: The majority of the 27 available cases did not show obvious signs of disease (70.4%). As the liver was the most frequent location, palpable enlargement of the liver was the most common sign (14.8%), followed by abdominal mass (7.4%) and tenderness (7.4%).

Laboratory examination: Nearly one in five patients had no aberrant findings (26 case files were available). Elevated white blood cell (WBC) count accounted for the highest percentage of findings (34.6%); liver function abnormity (30.8%), elevated CRP (19.2%), and erythrocyte sedimentation rate (ESR) (19.2%) were also frequent. Other abnormalities included hypoalbuminemia (15.4%), elevated tumor markers (15.4%), and anemia (15.4%).

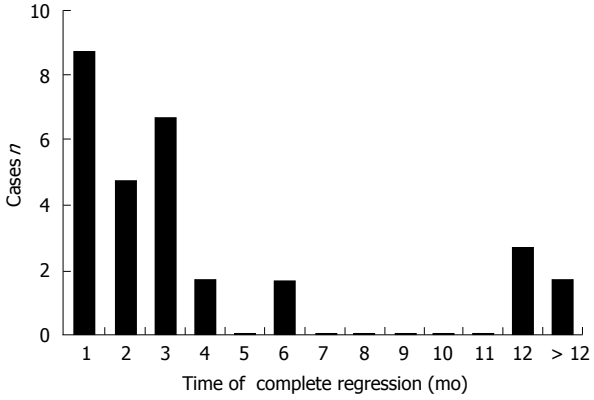

Figure 5 Time of complete regression of reported cases. The majority of cases reported that the mass shrunk within 3 mo, and almost all patients had complete resolution in 1 year.

Location: The liver was the most common location, making up 71.8% of all the reported cases. Retro-peritoneum, mesentery, gastrointestinal tract, and pancreas were also reported locations.

Size: The maximum diameter of the tumor was reported in 21 cases, and ranged from 1 cm to 15 cm. The average size was 6.1 cm.

Diagnosis: A total of 31 cases were diagnosed before treatment. Among them, 25 cases were diagnosed as IMT or IPT (80.6%) and led to conservative therapy. Nevertheless, 5 cases were diagnosed as malignant tumors, and so abdominal exploration was performed. All hepatic lesions were diagnosed before treatment, with the accuracy reaching 95.7%. However, only 77.8% of the masses located at the retro-peritoneum and mesentery could be diagnosed, and more than half of the masses were misdiagnosed as malignant tumors.

Management: Seventeen out of 38 cases did not receive any intervention *vs* 21 out of 38 cases that were managed medically. The most commonly used therapy was NSAIDs (15.8%), followed by steroids (13.2%) and antibiotics (10.5%). Of the remaining cases, 3 patients received a combination of medicines.

Outcome: All 38 patients were reported as thriving during the follow-up period. Thirty reports described the specific time that the tumor regressed, ranging from a few days to 3 years (Figure 5).

DISCUSSION

Although IMT and IPT were formerly regarded as the

same disease, some authors argue they are distinct entities. As there is no uniform view at present, the use of these two terms synonymously in the literature has led to confusion in understanding the incidence and behavior of the disease. In this article, we still attribute IMT to the broad category of IPT for now. IMT was initially identified in the lungs in 1937. Since then, it has been described at various sites[12]. As a result of various origins, IMT may present with diverse clinical symptoms, physical signs, and laboratory findings, which makes it difficult to differentiate from other neoplasms[21]. Radiological appearance is nonspecific and insufficient to make a correct diagnosis. At present, percutaneous biopsy is considered the most useful tool. Meis *et al*[25] reported that most cases of IMT expressed actin, vimentin, and keratin. Chan *et al*[26] speculated that the expression of ALK may be a specific marker for IMT. However, a biopsy is unable to provide an absolutely certain diagnosis[21], especially for tumors located at the retro-peritoneum. Therefore, we recommend laparoscopic exploration first for these undiagnosed cases. Large samples taken during a laparoscopic biopsy may increase the reliability of the histopathology results.

The reasons for the spontaneous regression of IMT have not been clearly elucidated; however, by analyzing the published cases, we can generate some hypotheses for further investigation.

First, different tumor locations might influence the prognosis of IMT. Hepatic lesions have the most favorable outcome; in contrast, tumors occurring in the abdomen, pelvis, and retro-peritoneum tend to show more aggressive behavior and have a poor prognosis[6,27]. Location is also associated with age and gender. The mean age of patients with IMT is reported to be as low as 9.7 years, and females account for approximately 60% of patients. By contrast, liver lesions usually develop in men of middle or advanced ages[22].

Second, regression cases are more frequently observed in middle-aged and older patients. Therefore, age may have an impact on prognosis. Studies showed that ALK positivity was detected in 36% to 60% of cases and was associated with cytogenetic abnormalities[28] and local recurrence[27]. Interestingly, the *ALK* gene was expressed selectively in younger patients[26] and may contribute to the unfavorable prognosis of children and adolescents.

Third, recurrence is related to a number of factors, such as aneuploidy, atypia, and ganglion-like cells. Coffin *et al*[29] demonstrated that nearly 75% of aneuploid IMT had recurrence and malignant transformation. Hussong *et al*[30] reported that atypia and ganglion-like cells were detected much more in cases with recurrence and malignant transformation.

As discussed above, we conclude that the prognosis of IMT is closely related to location, age, and ALK positivity. This is also the area of dispute regarding the diagnosis of IMT and IPT. Vecchio suggested the IPT, rather than IMT, should be seriously considered when managing ALK-negative spindle cell lesions in adult patients[31]. Gleason also agreed that one should be wary of making the diagnosis of IMT in these populations and recommended taking many factors into consideration, including age, location, histological pattern, and ALK expression[32]. We deem that both IPT and IMT must be subdivided into different types according to location, age, histology, gene expression, and chromosome condition. Different types may have dramatically different outcomes and, as a result, the therapeutic approach should be determined accordingly.

Complete resection has been widely accepted as the mainstay treatment, although surgery could be destructive to adjacent structures and increase morbidity[2]. Adjuvant chemotherapy in conjunction with radiation therapy is also controversial[8,33].

With more spontaneous regression cases being described, there is an increasing realization of conservative management[10,13,34]. Although some articles have reported the recurrence[6] or malignant transformation[35,36] of IMT, Goldsmith *et al*[23] compared the mortality rate of 215 liver IMT patients between medical management and resection surgery and found no significant difference. In terms of conservative options, there is no obvious difference in overall outcome between patients receiving antibiotics, steroids, or no treatment[23].

Although conservative therapies have been effective for a number of patients, these treatments are all empirical, and the mechanisms are not well understood. Some recent studies[8,34] reported that the majority of IMT tissues were positive for COX-2 and VEGF staining, and proposed that NSAIDs could have an anti-angiogenic effect via the inhibition of COX-2. Others[22] found that many plasma cells infiltrating at IMT lesions were positive for IgG4, and speculated that IMT was associated with an immune process. Therefore, the administration of steroids might prevent an immune response and reduce systemic symptoms[10].

To date, the optimal treatment of IMT is far from conclusive and needs to be further studied. We agree that resection should be the initial treatment for those patients with mass effect symptoms, those where the tumor mass tends to increase or is easily excised, and those in which a certain diagnosis cannot be made by biopsy[21,22]. However, when the mass is assessed as unresectable by a CT scan or laparoscopic exploration, surgical procedures should be avoided and conservative therapy with antibiotics, steroids, NSAIDs, or observation along with intense follow-up, should be taken into consideration[2].

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We thank the pathologists and the radiologists in our hospital for their technical assistance.

COMMENTS

Case characteristics

The symptoms of two middle-aged male patients were variable; one presented with abdominal pain and vomiting, and the other with abdominal distension, poor appetite, and weight loss.

Clinical diagnosis
The physical signs of the two cases were also variable. A large mass was palpated in the epigastrium for the first patient, while mild abdominal tenderness was observed during examination for the second.

Differential diagnosis
Malignant tumors (angiosarcoma, cystadenocarcinoma, and metastatic tumors), benign neoplasms (focal nodular hyperplasia, hemangioma, and adenoma), and abscesses.

Laboratory diagnosis
The first patient had an elevated neutrophil count (6.7 × 10^9/L), C-reactive protein (27.9 mg/L), lactic dehydrogenase (383 U/L), and CA125 (44.8 IU/mL), while the second had no remarkable abnormal laboratory results.

Imaging diagnosis
A computed tomography scan showed a large mass located in the abdominal cavity in both cases.

Pathological diagnosis
Histological examination showed a proliferation of myofibroblasts and an infiltration of inflammation cells. IHC staining showed CD34, desmin, and anaplastic lymphoma kinase (ALK) were negative, while SMA was positive in both cases.

Treatment
Neither patient received curative resection or further treatment.

Related reports
Spontaneous regression of an intra-abdominal inflammatory myofibroblastic tumor is seldom reported. The clinical and pathological characteristics of inflammatory myofibroblastic tumor are unclear and the treatment is controversial.

Experiences and lessons
This case report represents the clinical characteristics of intra-abdominal inflammatory myofibroblastic tumors and also discusses the treatment of inflammatory myofibroblastic tumors. We recommend that conservative therapy should be considered when the tumor is unresectable, especially for middle-aged patients with negative ALK expression.

Peer review
The authors have described two cases of intra-abdominal inflammatory myofibroblastic tumor which showed spontaneous resolution without intervention. The article highlights an important point which has important therapeutic implications.

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CASE REPORT

Long-term survival after enucleation of a giant esophageal gastrointestinal stromal tumor

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Author contributions: Mu ZM and Ye YW contributed to the manuscript writing and revision; Mu ZM, Xie YC and Peng XX did the surgery; all authors gave their approval in publishing this version of the manuscript.

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Key words: Esophageal gastrointestinal stromal tumor; Long-term survival; Enucleation; Surgery; Follow-up

Core tip: We report a case of giant esophageal gastrointestinal stromal tumor in a 29-year-old male successfully treated with enucleation. The patient has no evidence of recurrence and is in good clinical conditions up-to date, five years after surgery.

Mu ZM, Xie YC, Peng XX, Zhang H, Hui G, Wu H, Liu JX, Chen BK, Wu D, Ye YW. Long-term survival after enucleation of a giant esophageal gastrointestinal stromal tumor. *World J Gastroenterol* 2014; 20(37): 13632-13636 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i37/13632.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i37.13632

Abstract

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal neoplasms of the gastrointestinal tract. Less than 1% occurs in the esophagus. Surgery is the primary treatment for patients with GISTs. We report a 29-year-old male was admitted after the detection of a posterior mediastinal mass during work-up with routine examination. He did not have any disease-related symptoms. The physical examination was unremarkable. Chest computed tomographic scan, the barium esophagogram and endoscopic esophageal ultrasound showed benign neoplasm. The patient was performed an enucleation surgery through the right posterolateral thoracotomy. The pathology revealed a 13.0 cm × 12.0 cm × 5.0 cm mass. The tumor was CD117 (C-kit), PDGFRA and DOG1 positive. These findings were consistent with a GIST of the esophagus. So the diagnosis of GIST of esophagus was confirmed. The pathological diagnosis of low grade of GIST of esophagus was confirmed. The patient has no evidence of recurrence and is in good clinical conditions up-to date, five years after surgery.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal neoplasms of the gastrointestinal tract. The most frequent anatomic location of GIST is the stomach (60%-70%), followed by small intestine (20%-25%), colon and rectum (5%), and esophagus (< 1%)[1]. They typically present in older individuals. Surgery is the initial treatment for the esophageal GISTs. We present a rare case of long-term and good quality of life after enucleation of a giant esophageal GIST.

CASE REPORT

A 29-year-old male was admitted to department of thoracic surgery after the detection of a posterior mediastinal mass during work-up with routine examination. He did not have any disease-related symptoms, such as dysphagia, chest pain or dyspnea. The physical examination was unremarkable. Results of laboratory studies were all within normal limits. Chest computed tomographic (CT) scan revealed a 13 cm hyperdense soft-tissue mass,

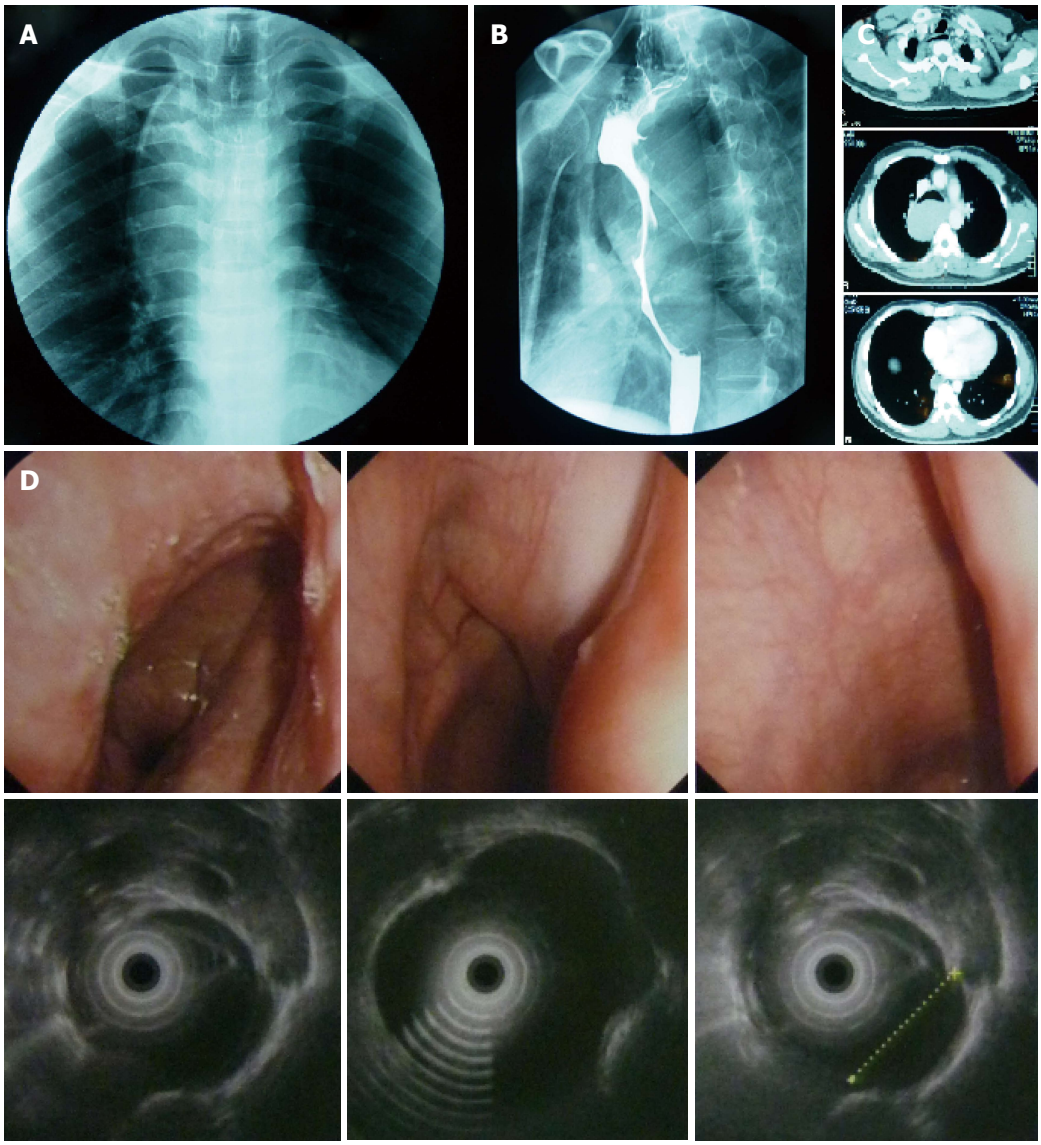
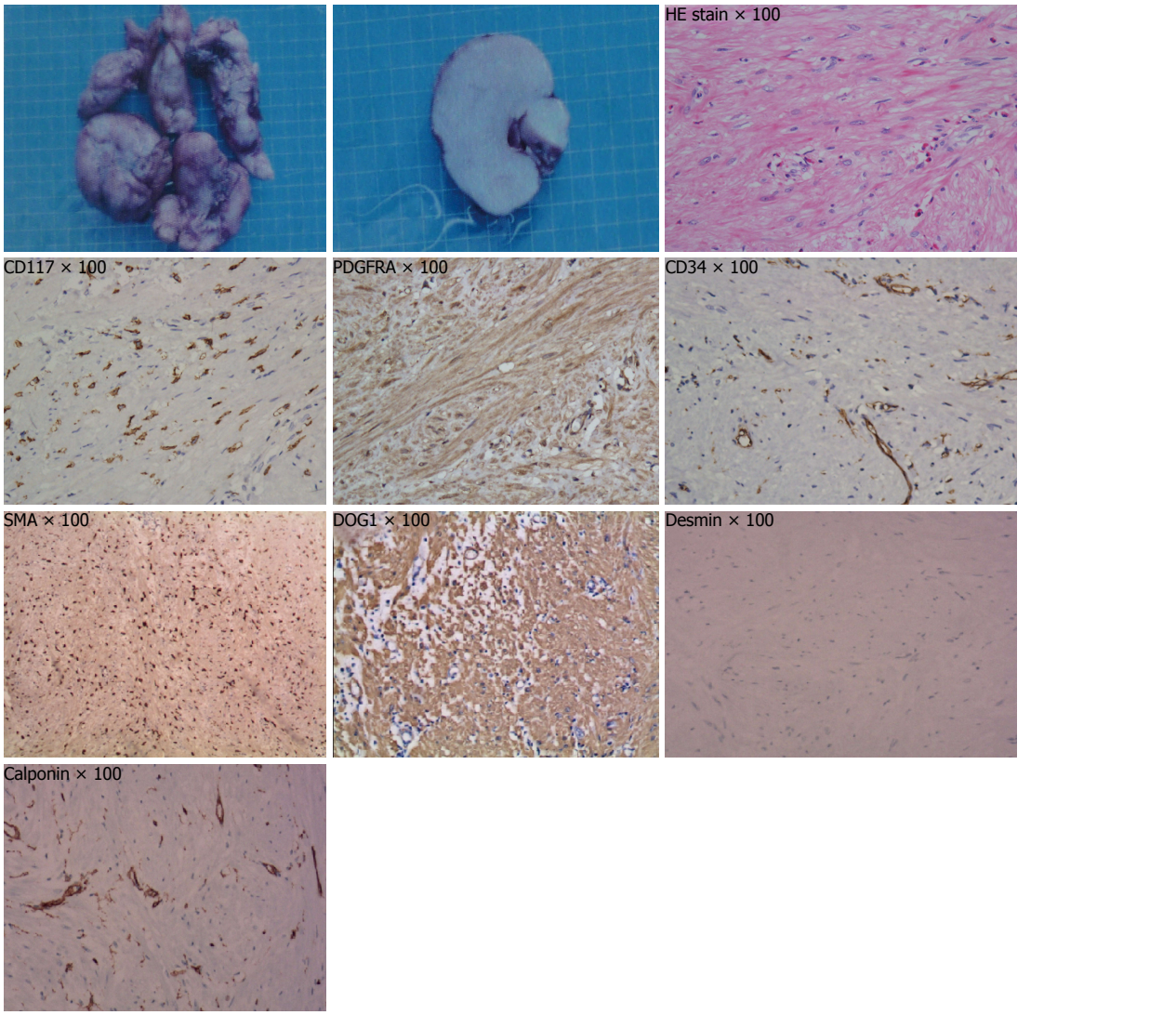


Figure 1 Mass showed before the surgery. A: The frontal chest radiograph showed a widened and undulated mediastinal contour; B: Barium esophagogram demonstrated a multilobulated protuberant lesion with a relatively smooth margin in the middle thoracic esophagus; C: Chest CT scan revealed a 13 cm × 5 cm × 6 cm hyperdense soft-tissue mass, originated from the posterior wall of the esophagus. The mass was well demarcated from the surrounding structures protruded into the esophageal lumen and compressed the trachea and the right main bronchus, and no metastases noted was found; D: ESU demonstrates a hypoechoic, homogeneous, well-demarcated mass, arising at 24 cm from the incisors and extending to 35 cm and arising from the muscularis propria of the esophagus.

originating from the posterior wall of the esophagus. The barium esophagogram demonstrated a multilobulated protuberant lesion with a relatively smooth margin in the middle thoracic esophagus. Endoscopic esophageal ultrasound (EUS) demonstrated a hypoechoic, homogeneous, well-demarcated mass, arising from the muscularis propria of the esophagus at 24 cm from the incisors and extending to 35 cm (Figure 1).

Preoperative examination and intraoperative findings showed benign neoplasm. The patient was underwent an enucleation surgery through the right posterolateral thoracotomy. The outer esophageal muscle was incised longitudinally. Careful dissection was done to separate and remove the mass from the underlying submucosa. After careful dissection, the mass was enucleated completely with unbroken pseudocapsule. During the operation, one piece of mucosa was inadvertently opened during dissection, which was repaired by interrupted suture. The underlying mucosa and longitudinal muscle were reapproximated. The muscularis propria and mediastinal pleural also were sutured. Intraoperative endoscopy with air insufflation was used to confirm mucosal integrity and safeguard against esophageal perforation.

The pathology revealed a 13.0 cm × 12.0 cm × 5.0 cm mass with an intact pseudocapsule. The tumor was CD117 (C-kit), PDGFRA, DOG1 and SMA positive, CD34 and calponin and weak positive, and negative for desmin. These findings were consistent with a GIST of the esophagus. The mass contained less than 5 mitoses per 50 high-power fields. So the diagnosis of low grade of GIST of esophagus was confirmed. We also found that exon 12 and exon 18 of PDGFRA and exon



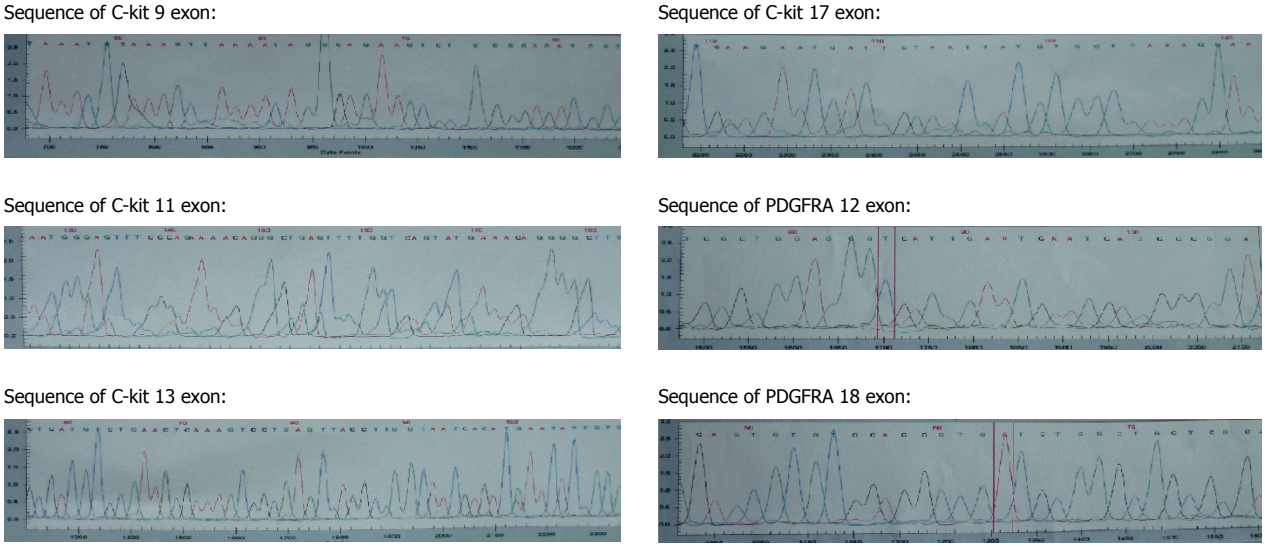


Figure 2 Pathology revealed a 13.0 cm × 12.0 cm × 5.0 cm mass with an intact pseudocapsule. The tumor was CD117 (C-kit), PDGFRA, DOG1 and SMA positive, CD34 and calponinand weak positive, and negative for desmin. Mutation sequencing found that exon 12 and exon18 of PDGFRA and exon 9,11,13,17 of C-kit all were wild type.

9,11,13,17 of C-kit all were wild type (Figure 2).

In one week postoperatively, the patient underwent an esophagogram that confirmed the absence of an esophageal leak or stricture. The patient had liquid diet one week

after operation, swallowed most ordinary food with little difficulty and had no dysphagia two weeks later. At 5-year follow-up the patient is feeling well and CT scans have shown no evidence of recurrent disease.

DISCUSSION

GISTs are uncommon mesenchymal tumors of the gastrointestinal tract and rare before the age of 40 years, and less than 1% occur in the esophagus[1,2]. Bülbül Doğusoy *et al*[3] reports a multicenter study of 1160 gastrointestinal stromal tumors and found 0.7% to be located in the esophagus.

The diagnosis of esophageal GIST is frequently not clear preoperatively. Half of patients with esophageal GIST are asymptomatic and the remaining ones present with dysphagia or atypical chest pain. A complete diagnostic evaluation includes esophagoscopy, EUS, CT scan, and barium esophagogram. The presence of a rounded filling defect on barium esophagogram, an endoscopically normal-appearing mucosa, a well-circumscribed, non-enhancing mass on contrast -enhanced CT scan, and a homogeneous, hypoechoic mass with smooth borders on EUS examination will help in the differential diagnosis with other malignant neoplasms but not with esophageal leiomyoma. CT can be helpful in the preoperative assessment of tumor extent and presence of distant metastases. A definite diagnosis of GIST depends on a comprehensive pathologic examination of the surgical specimen. In GIST cases, approximately 95% are KIT (CD117)-positive, CD34 is positivity in 95% and SMA is positive in 20%-30%, but often nonreactive with S-100 protein immnostains[4]. CD117 and DOG1 positive are specific in GIST as compared with smooth muscle and neural tumors. In the case represented here, we diagnosed the mass was GIST mainly because of positive staining for CD117 and DOG1.

There were few effective therapeutic options for patients with GISTs. For patients with primary localized and resectable GIST, surgical resection remains the only chance for cure, as chemotherapy and radiation are ineffective. The primary goal of surgical management of GISTs is to achieve a negative microscopic margin[5,6].

The surgery options available for esophageal GISTs are fairly controversial, esophageal resection or GIST enucleation.

Since a true tumor capsule does not exist, the surgeon must exercise additional care during the removal of a GIST not to rupture the tumor, as this complication is associated with an increased risk for the development of peritoneal metastases[7]. Much like with most other soft tissue sarcomas, GISTs rarely metastasize to lymph nodes, thus a regional lymphadenectomy is not necessary, unless locoregional lymph nodes are enlarged[8-10]. It is extremely important to avoid tumor rupture in the surgery because it is associated very poor outcomes. The tumor should not be held with forceps and should be handled gently. If there is any possibility of tumor rupture, an en bloc combined with a resection or even abandoning surgery and converting to neoadjuvant treatment should be considered. In the case present, we sutured and then draged the tumor through the stitch during the operation to avoid rupturing the tumor. All the procedures in the enucleation were out of pseudocapsule. We must make sure that the enucleated tumor with unbroken pseudocapsule negative margins.

Activating mutations of KIT or platelet-derived growth factor receptor alpha (PDGFRA) are found in the vast majority of GISTs. Two KIT and PDGFRA kinase inhibitors, imatinib and sunitinib, have proven clinical benefit in patients with recurrent or metastatic GISTs and have been received approval from the FDA for the treatment of gastrointestinal stromal tumours. More than half of patients unfortunately develop resistance to imatinib after about 2 years, eventually develop resistance to these agents, resulting in fatal disease progression[11,12]. Some other structurally distinct inhibitors of KIT and PDGFRA kinases have been developed, Demetri *et al*[13] showed that oral regorafenib could provide a significant improvement in progression-free survival compared with placebo in patients with metastatic GIST after failure of imatinib and sunitinib. Ajuvant tyrosine-kinase inhibitor treatment is debated as its survival benefit is controversial. Blay *et al*[5] reported that patients with completely resected GISTs who received adjuvant imatinib after surgery had not improvement the overall survival, although lower recurrence rates. Patients with wild-type GISTs are less responsive to imatinib-based therapies. In the case presented here, the patient was wild-type GIST, so he didn't take the adjuvant treatment.

The 5-year survival rate after complete resection of GISTs is approximately 50%-65%. Many factors including tumor size, mitotic rate, tumor location, kinase mutational status and occurrence of tumor rupture have been extensively studied and proposed to be predictors of survival outcomes. Tumor size and mitotic activity are the best prognostic features. Tumors are classified using a ranking system, grouping tumors into very low-, low-, intermediate-, and high-risk categories based on size (< 2 cm, 2-5 cm, 5-10 cm, and > 10 cm) and on number of mitoses within 50 high-power fields (HPFs); such measurements typically being reported as less than 5, 5 to 10, or greater than 10[14]. In the case represent here, the tumor size and the mitotic activity expressed per 50 HPFs were 13 cm and less than 5, respectively.

Katoh *et al*[15] reported 5 cases of GIST, and conclusion that if low malignancy GIST is completely removed by endoscopic resection, then no further treatment is necessary. In the case presented here, the patient did not receive any adjuvant therapy after surgery and no evidence of recurrent disease.

In the case presented here, the patient was diagnosed with a giant esophageal gastrointestinal stromal tumor in 29 years old. We also found that exon 12 and exon18 of PDGFRA and exon 9, 11, 13, 17 of C-kit all were wild type. These findings were consistent with a GIST of the esophagus. The mass contained less than 5 mitoses per

50 high-power fields. So the diagnosis of low grade of GIST of esophagus was confirmed. He did not receive any adjuvant therapy after surgery. At 5-year follow-up the patient is feeling well and CT scans have shown no evidence of recurrent disease.

COMMENTS

Clinical diagnosis

Computed tomographic (CT) the barium esophagogram; esophageal ultrasound (EUS) showed benign neoplasm from esophagus.

Differential diagnosis

In contrast to gastrointestinal stromal tumors (GISTs), leiomyoma and leiomyosarcoma are positive for SMA and desmin and negative for KIT and CD34.

Imaging diagnosis

Chest CT scan, the barium esophagogram and endoscopic EUS showed benign neoplasm in esophagus.

Pathological diagnosis

The authors do the immunohistochemistry for CD117, PDGFRA, DOG1, CD34, smooth muscle actin, desmin and calponin. We found that positive for CD117, PDGFRA, DOG1, CD34 and smooth muscle actin, negative for desmin and calponin.

Treatment

The authors enucleated a giant esophageal gastrointestinal stromal tumor in a 29-year-old male.

Related reports

Milman S reported enucleation of a giant esophageal gastrointestinal stromal tumor in an old man in 2009.

Experiences and lessons

The authors can enucleated a giant esophageal gastrointestinal stromal tumor and have good prognosis.

Peer review

The authors diagnosed the GISTs after the surgery, maybe we could diagnosed through biopsy or other methods before the surgery.

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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