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EDITORIAL

Surgery for inflammatory bowel disease in the era of laparoscopy

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Abstract

During the course of inflammatory bowel disease (IBD), surgery may be needed. Approximately 20% of patients with ulcerative colitis (UC) will require surgery, whereas up to 80% of Crohn's disease (CD) patients will undergo an operation during their lifetime. For UC patients requiring surgery, total proctocolectomy and ileoanal pouch anastomosis (IPAA) is the operation of choice as it provides a permanent cure and good quality of life. Nevertheless a permanent stoma is a good option in selected patients, especially the elderly. Minimally invasive surgery has replaced the conventional open approach in many specialized centres worldwide. Laparoscopic colectomy and restorative IPAA is rapidly becoming the standard of care in the treatment of UC requiring surgery, whilst laparoscopic ileo-cecal resection is already the new gold standard in the treatment of complicated CD of terminal ileum. Short term advantages of laparoscopic surgery includes faster recovery time and reduced requirement for analgesics. It is, however, in the long term that minimally invasive surgery has demonstrated its superiority over the open approach. A better cosmesis, a reduced number of incisional hernias and fewer adhesions are the long term advantages of laparoscopy in IBD surgery. A reduction in abdominal adhesions is of great benefit when a second operation is needed in CD and this influences positively the pregnancy rate in young women undergoing restorative IPAA. In developing the therapeutic plan for IBD patients it should be recognized that the surgical approach to the abdomen has changed and that surgical treatment of complicated IBD can be safely performed with a true minimally invasive approach with great patient satisfaction.

Key words: Laparoscopy; Ulcerative colitis; Surgery; Inflammatory bowel disease; Laparoscopic surgery; Proctocolectomy; Ileoanal pouch anastomosis

Core tip: The clinical management of inflammatory bowel disease (IBD) patients has dramatically changed in the last decades and primary, secondary and even tertiary levels of medical treatment are available to treat both Crohn's disease or ulcerative colitis. However, it should be recognized that surgical approaches have also changed, for the better, in the last few years and that minimally invasive surgery is now available in most centers. The timing of surgery is a key issue for proper management of IBD patients. Laparoscopic surgery should be seen as less aggressive than the standard surgical approach and could lower the threshold for surgical intervention.

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INTRODUCTION

During the course of inflammatory bowel disease (IBD),

surgery may be needed. Approximately 20% of patients with ulcerative colitis (UC) will require surgery, whereas up to 80% of Crohn's disease (CD) patients will undergo an operation during their lifetime[1]. For UC patients requiring surgery, total proctocolectomy is the operation of choice as it provides a permanent cure and ileoanal pouch anastomosis (IPAA) has replaced the classic permanent ileostomy as the procedure of choice to accompany a proctocolectomy. Partial colectomy is rarely performed because of the high probability that the disease will recur in the remaining colon. Nevertheless partial colectomy and ileo-rectal anastomosis as well as proctocolectomy and permanent ileostomy are still good options in selected patients, especially the elderly.

For CD, surgery is not a definitive cure. Therefore, intestinal resection is indicated for patients who are refractory to the therapy or who are intolerant to medical treatments. In addition, patients that show severe complications of the disease will require surgery for obstruction, recurrent sub-obstructions, abdominal abscesses, perforation, massive bleeding or even cancer. The most common surgical procedure is ileo-cecal resection and primary reconstruction, which is indicated in patients with CD of distal ileum and/or ileo-colon. Strictureoplasty is less frequently indicated in patients with limited proximal small bowel strictures. Endoscopic dilatations of jejunum and ileum and more limited resections are also employed in a minority of cases. Endoscopic recurrence 1 year after ileo-colonic resection is observed in up to 80% of patients, while clinical recurrence is observed in about 20% of patients at 2 years and in up to 80% at 20 years[2].

There is little question that the timing of surgical intervention is a key issue for proper management of IBD patients. Indication and timing of surgery in IBD requires a joint evaluation by dedicated gastroenterologists and surgeons.

IBD surgery can be regarded as a cure for UC whilst it is undertaken to improve symptoms and to ameliorate the quality of life for CD patients. In both UC and CD, surgery can be a salvage procedure for acute, severe disease.

Although gastroenterologists are familiar with the potential complications associated with acute severe disease, most are reassured by statistics consistently showing an overall standardized mortality ratio for IBD patients near to that of general population[3]. A large scale analysis performed in the Oxford region (United Kingdom) using record linkage studies, showed that 3-year mortality was significantly lower among people who underwent elective colectomy for IBD than among those who were admitted to hospital without colectomy or who had had emergency colectomy[4]. The most worrying finding of the study by Roberts and colleagues is that in patients admitted for UC and CD who had no surgery (13.6% and 10.1% respectively) most deaths occur between 6 mo and 36 mo after admission. The decision to operate is an important one and should be made after a careful evaluation of all the clinical variables in each individual patient at the time of the diagnosis of the disease. Undoubtedly, many people suffer needlessly because they try to avoid surgery. Surgical delay does not only put the patient through unnecessary periods of pain and suffering, but it can also increase the risks of operative complications and ultimately lead to a worse outcome. On the other hand, it is clear that avoiding or delaying surgery may be the better choice for IBD patients, particularly in young CD patients, because of the almost certain recurrence of the lesions.

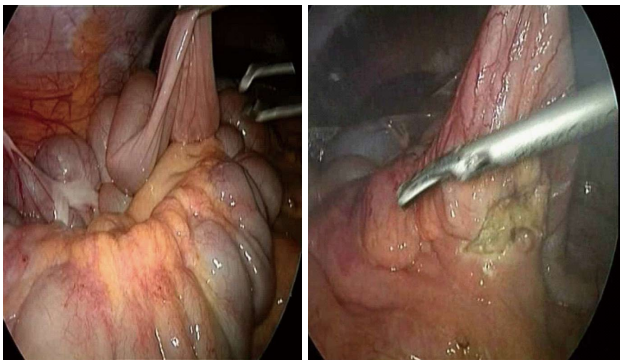

Figure 1 Laparoscopic ileo-cecal resection.

The general belief that surgery for IBD should be the last resort is flawed by reports on outcomes after colectomy in IBD patients, showing a relatively good prognosis[2,5-7]. However, these reports are mainly small, short term studies from specialist centers, not taking into account data from district hospitals where operations are performed mainly in emergency situations or with less positive results.

Furthermore the development of the therapeutic plan for IBD patients should also take into account the fact that the surgical approach to the abdomen has changed in recent years. Colectomies can be safely performed using a minimally invasive approach[8] to the great satisfaction of the patient (Figure 1). Most CD patients who have undergone laparoscopic ileo-cecal resection have been reported to choose a laparoscopic operation should the disease recur[9].

This is in agreement with data from the bariatric surgery where a steep increase in the request for surgical treatment of morbid obesity was observed after the development of minimally invasive procedures.

Surgery is certainly not the cure for CD, but is a viable therapeutic option and, given the potential advantages of the minimally invasive surgery, it shouldn't be always put at the top of the pyramid of treatment. In selected subgroups of patients, early surgery is correlated with a more favorable surgical outcome and a laparoscopic ileo-cecal resection together with a fast track recovery protocol[10] may represent an appealing alternative to several years of medication. There is currently enough evidence to suggest a laparoscopic ileo-cecal resection as the gold standard in the management of CD patients with obstructive symptoms, but no significant evidence of active inflammation[11]. In fact, this group of patients (less than 40 cm affected bowel and appreciable symptoms but no imminent obstruction) respond well to medical treatment

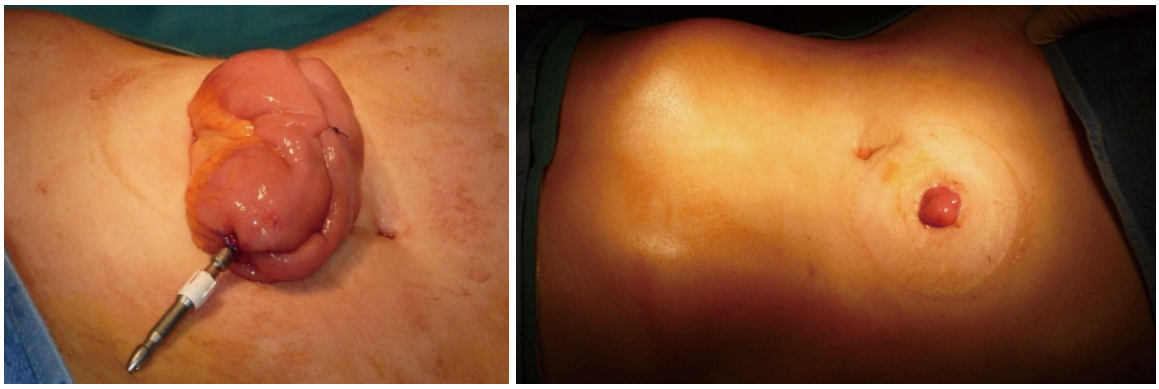

Figure 2 Totally laparoscopic proctocolectomy and restaurative ileoanal pouch anastomosis.

but will almost always require surgery during the course of their disease. A delayed surgical approach may not only increase the risk of septic complications but it may also reduce the possibility of performing a minimally invasive operation. CD patients who have undergone years of medication, and who present with a severely thickened bowel and mesentery due to marked inflammation and fibrosis, are candidates for more extensive resection with little chance of undergoing a minimally invasive procedure.

The quality of life for patients with UC is improved after colectomy[2]. Surgery is indicated in UC when medical therapy is ineffective, intractability being one of the most common reasons for proctocolectomy. Alternatively, steroid dependence which is not responsive to immunomodulatory drugs (including biologics), low compliance, multifocal dysplasia or high grade dysplasia, may represent indications for surgery. As for CD, delaying surgery may increase mortality and morbidity in UC. In patients who have undergone an emergency colectomy for UC, the risk of death increases substantially in the four to six months after surgery[4]. It has been recommended that about 85% of patients who do not respond to conventional steroid treatment within 6 d of hospitalization should undergo colectomy. A subgroup of these patients could alternatively be treated with anti-tumor necrosis factor "rescue" therapy, in accordance with the European Crohn and Colitis Organization guidelines[12]. Nevertheless, all of these criteria need to be considered in the light of the clinical characteristics of each individual patients, and a possible decision for surgery needs to be jointly assessed by an experienced gastroenterologist and surgeon on a daily basis. Furthermore, in UC the timing of surgery influences the surgical approach and vice versa: an emergency colectomy usually ends with a terminal ileostomy. This procedure is followed, generally after several months, by a completive proctectomy, restaurative pouch and lateral ileostomy. The third and last operation, the ileostomy closure, will be performed after a few months, provided the good condition of the ileo-anal pouch. However, in cases of planned elective surgery it will be possible to avoid one operation by performing a totally laparoscopic procto-colectomy and IPAA at the same time, followed by the closure of the lateral ileostomy (Figure 2).

Optimal care of patients with IBD continues to involve a great deal of judgment. Avoiding mortality and achieving a good quality of life are the guiding principles in the care of IBD patients. The decision to operate remains a difficult one and should take into account all the pros and cons of a planned "nice" laparoscopic resection compared to the symptomatic relief that may be achieved by primary, secondary or even tertiary medical therapy. Randomized controlled trials that include a large number of patients are required to establish the optimal timing for surgery in IBD.

Finally, but most importantly, unless severe complications indicate the need for emergency surgery, a decision for elective surgery must take into account not only the clinical characteristics of each patients but also the view of the patient. Indeed, the timing of surgery needs to be extensively discussed and approved not only by the gastroenterologist and surgeon, but also by the patient, who should be clearly informed of the risks and benefits of both medical and surgical therapy.

Laparoscopic surgery in IBD is safe and feasible. It offers both cosmetic advantages and some short term advantages, such as a possible reduction in perioperative complications[13]. Long term advantages include fewer incisional hernias and fewer adhesions[14] with a significant impact on female fertility in UC patients[15]. The minimally invasive procedure is the approach that is preferred in specialized centres. Considering its proven advantages and popularity amongst patients, it should be seen as a new strategic option when considering therapeutic alternatives in complicated IBD patients.

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TOPIC HIGHLIGHT

Eleftherios Tsiridis, PhD, *Series Editor*

Genetic association of interleukin-6 polymorphism (-174 G/C) with chronic liver diseases and hepatocellular carcinoma

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Author contributions: Giannitrapani L and Soresi M collected and analyzed literature data; Balasus D and Licata A contributed analytic tools; Montalto G supervised the paper and Giannitrapani L wrote the paper.

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Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine which is expressed in many inflammatory cells in response to different types of stimuli, regulating a number of biological processes. The *IL-6* gene is polymorphic in both the 5′ and 3′ flanking regions and more than 150 single nucleotide polymorphisms have been identified so far. Genetic polymorphisms of *IL-6* may affect the outcomes of several diseases, where the presence of high levels of circulating IL-6 have been correlated to the stage and/or the progression of the disease itself. The -174 G/C polymorphism is a frequent polymorphism, that is located in the upstream regulatory region of the *IL-6* gene and affects IL-6 production. However, the data in the literature on the genetic association between the -174 G/C polymorphism and some specific liver diseases characterized by different etiologies are still controversial. In particular, most of the studies are quite unanimous in describing a correlation between the presence of the high-producer genotype and a worse evolution of the chronic liver disease. This is valid for patients with hepatitis C virus (HCV)-related chronic hepatitis and liver cirrhosis and hepatocellular carcinoma (HCC) whatever the etiology. Studies in hepatitis B virus-related chronic liver diseases are not conclusive, while specific populations like non alcoholic fatty liver disease/non-alcoholic steatohepatitis, autoimmune and human immunodeficiency virus/HCV coinfected patients show a higher prevalence of the low-producer genotype, probably due to the complexity of these clinical pictures. In this direction, a systematic revision of these data should shed more light on the role of this polymorphism in chronic liver diseases and HCC.

Key words: Single nucleotide polymorphisms; Interleukin-6; Chronic hepatitis; Liver cirrhosis; Hepatocellular carcinoma

Core tip: Several studies suggested the possibility of an association between -174 interleukin-6 gene G/C polymorphism and some liver diseases however, the data in the literature are still controversial. This work aims to review the literature data on the role of this polymorphism and its possible biological function in chronic liver diseases and hepatocellular carcinoma.

Giannitrapani L, Soresi M, Balasus D, Licata A, Montalto G. Genetic association of interleukin-6 polymorphism (-174 G/C) with chronic liver diseases and hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(16): 2449-2455 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i16/2449.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i16.2449

INTRODUCTION

In recent decades chronic liver diseases (chronic hepatitis, liver cirrhosis) and hepatocellular carcinoma have become more and more diffuse both in Western and in

Eastern countries, representing an important problem for health systems worldwide[1-5]. Whatever the etiology, these diseases share a common pathogenetic mechanism which is linked to chronic inflammation[6]. Hepatotropic viruses, toxins and alcohol, metabolic liver disease or autoimmunity can be the triggers which, acting chronically in the liver, ultimately activate cellular pathways involving transcription factors of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family and signal transducer and activator of transcription 3 (STAT3), as well as cytokines such as interleukin-6 (IL-6) and IL-1α, *etc*.

In particular, IL-6 is a cytokine involved in the regulation of several cellular processes including proliferation and differentiation and plays a pivotal role in acute phase response and in the control of the balance between pro-inflammatory and anti-inflammatory pathways. The *IL-6* gene is located on chromosome 7p21[7]. A number of studies indicated that the presence of a G/C single nucleotide polymorphism (SNP) at the promoter -174 of the *IL-6* gene, one of the numerous known polymorphisms in the *IL-6* gene, is related to the *IL-6* gene transcription rate and, as a consequence, to the control of circulating IL-6 levels[8,9].

Subsequently, two phenotypes for this polymorphism were identified: the high-producer phenotype, including the -174 G/G and -174 G/C genotypes, characterized by higher circulating IL-6 levels; and the low-producer phenotype, including the -174 C/C genotype[8]. Genetic population studies have shown that there are ethnic differences in the frequency of the -174 G allele, with higher frequencies in non-Caucasian than in Caucasian populations[10,11].

High circulating levels of IL-6 have been documented in several clinical conditions (inflammatory, neoplastic diseases) and in particular in various liver diseases such as viral chronic hepatitis[12], alcoholic liver disease[13], liver cirrhosis and hepatocellular carcinoma (HCC)[14]. A small number of studies have investigated a possible correlation between the presence of the -174 G/C polymorphism, IL-6 circulating levels and the stage of disease[15]. However, the results of these studies are quite controversial. This work aims to review the literature data on the role of G/C base exchange at position -174 of the *IL-6* gene and its possible biological function in chronic liver diseases and HCC.

IL-6 POLYMORPHISM (-174 G/C) AND HEPATITIS C VIRUS AND HEPATITIS B VIRUS INFECTION

Produced by a variety of cells such as macrophages, B and T cells and fibroblasts, IL-6 plays a central role in the inflammatory response associated with the course of chronic hepatitis due to hepatitis C virus (HCV)- and hepatitis B virus (HBV)-related infection[16,17].

To mediate its biological effects it interacts with a receptor complex consisting of a specific ligand-binding protein (IL-6R, gp80) and a signal transduction protein (gp130) (Figure 1A). When IL-6 binds its cell surface receptor (IL-6R) on the hepatocyte a homodimer of the signal transduction receptor gp130 is recruited to the complex and it activates a janus kinase 1 which in turn triggers two main signaling pathways: the gp130 Tyr759-derived Src homology 2 domain-containing protein tyrosine phosphatase-2/extracellular-signal-regulated kinase/mitogen-activated protein kinase pathway and the gp130 YXXQ-mediated Janus associated kinase/signal transducer and activator of transcription pathway (Figure 1B). Interestingly, sIL-6R (soluble form of IL-6R) also binds with IL-6, and the IL-6-sIL-6R complex can then form a complex with gp130[18,19]. Through this receptor system IL-6 can influence various cell types and exert its multiple biological activities regulating immune response, acute phase response and inflammation.

During HCV infection, an altered production of cytokines seems to be related to viral persistence and to affect response to therapy. Barret *et al*[20] comparing various cytokine polymorphisms (including -174 G/C *IL-6*) in individuals with spontaneous viral clearance after HCV infection and in those with persistent viremia, reported that the CC genotype with low IL-6 production was associated with spontaneous viral clearance, while an association between the high IL-6 producer genotypes and persistent infection only became apparent when both genotypes (GG and GC) were combined. As regards the influence of the genetic background in individuals with HCV infection and response to the antiviral therapy, the most recent literature data have investigated the role of *IL-28B* polymorphisms as a predictor of the outcome of the commonly-used treatments[21]. However, because of the central importance of IL-6 as a mediator of the immune response to infectious agents, and considering that host genetic variation, and in particular haplotypes, may affect IL-6 expression, Yee *et al*[22] examined the contribution of haplotypes in the *IL-6* gene to therapy for chronic HCV infection on sustained viral response (SVR). Among the SNPs genotyped and included in haplotype construction, the authors found some SNPs (including -174G/C, and in particular genotypes GG and GC) showing significant associations with a reduced likelihood of SVR.

These results are in contrast with previously reported ones published by Nattermanne *et al*[23] which, however, were obtained in another specific population, *i.e.,* patients co-infected with both acute and chronic HCV and human immunodeficiency virus (HIV). The aim of this study was to evaluate whether *IL-6* -174 G/C polymorphism could affect response to antiviral treatment in HCV-infected HIV-positive patients. The study group was compared to a group of HCV- and a group of HIV-monoinfected patients, as well as to a group of healthy individuals and no significant difference was found in the distribution of *IL-6* genotypes between the study groups. However, the authors concluded that carriers of high-producer genotypes (genotypes *IL-6* 174 GG and 174 GC) had significantly higher SVR rates than patients with an IL-6 low-

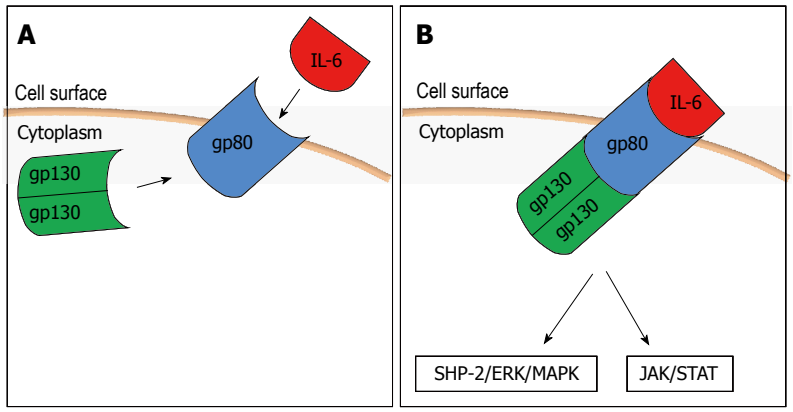

Figure 1 Interleukin-6 interaction with its receptor complex. A: Mechanism of action of the interleukin-6 (IL-6); B: Activation of the two pathways triggered by the IL-6 action. SHP-2: Src homology 2 domain-containing protein tyrosine phosphatase-2; ERK/MAPK: Extracellular-signal-regulated kinase/mitogen-activated protein kinase; JAK/STAT: Janus associated kinase/signal transducer and activator of transcription.

producer genotype (genotype 174 CC).

Another particular subgroup of HCV patients is the one with persistently normal or near normal alanine aminotransferases levels (PNALT), which for several years was supposed to have a milder course of disease, whereas it is now well known that in a few cases it can evolve to cirrhosis[24]. Among the studies evaluating genetic polymorphisms in chronic HCV carriers with PNALT, Falleti *et al*[25] evaluated the role of five *IL-6* polymorphisms (among them -174 G/C) in modulating fibrosis progression in PNALT patients with chronic HCV infection. The principal point of interest in this study were the associations found between *IL-6* polymorphisms and grading and staging increase during the follow-up of the patients with chronic viral hepatitis C and PNALT. In particular, grading increase appeared to be related to the presence of the G allele of the *IL-6* -174G/C polymorphism, while the C allele seemed to be protective.

As cytokines also play a fundamental role in the immune response to HBV and HBV infection may have different forms of evolution (self-limited or persistent and progressive), *IL-6* polymorphisms have also been studied to investigate a possible correlation between IL-6 promoter variants and chronic hepatitis B progression, infection evolution in adult patients and risk of HCC development. Unfortunately, the data reported by Park *et al*[26] are not conclusive because in their attempt to analyze additional polymorphisms in variants of genes implicated in chronic hepatitis B progression they found that Koreans and Caucasians had different genetic backgrounds in terms of the allele frequencies of the *IL-6* promoter SNPs. In particular, in their study the allele frequencies reported in Caucasians (range: 0.40-0.45) were much higher than those found in Koreans (allele frequencies 0.002). The authors concluded that at least in their population, although IL-6 may have important functions in the progression of chronic HBV infection, its genetic variants probably do not influence the development of LC and HCC from chronic HBV infection, due to too low frequencies of *IL-6* 174 G/C.

Another attempt to correlate cytokine genetic polymorphism with hepatitis B infection evolution was made in a Brazilian population, but the study found no significant differences in the polymorphism of *IL-6* -174 between the chronic HBV patient group and the self-limited infection group as regards alleles, genotypes or phenotypic expression[27].

Similarly, the study of a Japanese population by Migita *et al*[28] with the aim of characterizing cytokine gene polymorphisms in chronic HBV infection and their associations with HCC, was unable to show conclusive data about the role of *IL-6* -174 because no polymorphisms were found at that position (Table 1).

IL-6 POLYMORPHISM (-174 G/C) AND NON-VIRAL CHRONIC LIVER DISEASES

Non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) includes a broad spectrum of clinic-pathological entities, including simple steatosis and non-alcoholic steatohepatitis (NASH), which can progress to advanced liver diseases[29]. Its pathogenesis is strictly linked to insulin resistance and to all the mechanisms described for the development of metabolic syndrome, and in this perspective an important role is played by genetic background[30,31]. It is well known that the balance between pro- and anti-inflammatory acting cytokines is fundamental in the control of hepatic and systemic insulin action, and as a consequence, in the development of NAFLD. In particular, serum levels of this cytokine correlate remarkably well with the presence of insulin resistance, and adipose tissue-derived IL-6 has been shown to regulate hepatic insulin resistance *via* up-regulation of suppressor of cytokine signaling 3[32]. However, the role of -174 G/C polymorphism in this population raises some questions. In fact, a study by Carulli *et al*[33] found that the *IL-6* -174C variant, is significantly more prevalent in NAFLD than in healthy subjects, is associated with increased fasting insulin and homeostasis model assessment of insulin resistance, and is an independent predictor of NAFLD and NASH. This finding is in contrast with other studies which showed that the *IL-6* -174G variant was as-

sociated with lipid abnormalities[34] and with diabetes in Caucasians as well as Pima Indians[35-38] and that the C allele at -174 position was unlikely to play a role in the development of type 2 diabetes mellitus in a Taiwanese population[39]. One possible explanation for these contradictory results can be found in the conclusion of a study on an experimental mouse model of ASH and NAFLD: IL-10-/- mice were prone to liver inflammatory response but resistant to steatosis and hepatocellular damage induced by ethanol or high-fat diet feeding, thanks to the elevation of inflammation-associated hepatic IL-6/STAT3 activation that subsequently down-regulated lipogenic genes, but up-regulated fatty acid oxidation-associated genes in the liver[40].

Alcoholic liver diseases

In an attempt to explain why only a minority of heavy drinkers develop alcoholic liver cirrhosis or alcohol use disorders, some genetic factors have been considered[41,42], such as polymorphisms of genes encoding cytokines. Several studies support the hypothesis of a pivotal role of ethanol-induced cytokine changes in contributing to alcohol pathogenesis in a number of tissues, including the liver[43-45]. Moreover, elevated serum concentrations of pro-inflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-6 and IL-8 and decreased levels of anti-inflammatory cytokines like IL-10 have been shown in patients with this disease[43,46,47]. However, the only study on common polymorphisms in interleukin genes (including -174G/C *IL-6*) in a population of Spanish alcoholic patients did not find any statistically significant associations between any of the studied polymorphisms or the combinations of pro-inflammatory polymorphisms and the risk of alcoholic liver cirrhosis or alcohol abuse or dependence[48].

Autoimmune liver diseases

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis represent the three main categories of autoimmune liver diseases. However, their etiology and possible environmental triggers still remain obscure even if it is well established that a complex genetic background contributes to disease susceptibility and severity. Several studies have established that genetic factors are involved in the pathogenesis of autoimmune liver diseases[49-52]. Among these studies, one in a Chinese population of patients with AIH and PBC found that frequency of *IL-6* -174C was high and significantly increased in PBC patients compared with controls. This result supports the hypothesis that the *IL-6* -174G/C polymorphism could contribute to the change in susceptibility to PBC in some subjects[53] (Table 2).

IL-6 POLYMORPHISM (-174 G/C) AND HCC

An interrelation between chronic inflammation and cancer has been suspected for a long time[54]. Many tumors occur in association with chronic infectious diseases and persistent inflammation increases the risk and accelerates the development of cancer[55-59]. HCC is one of the most clear examples of inflammation-related cancer[60,61]. It is a tumor that slowly progresses through a chronic inflammation state, triggered by exposure to various agents. The molecular links that connect inflammation and cancer are not completely known, although there is a consistent body of evidence pointing to the role of transcription factors such as NF-κB[62] and STAT3[63] and cytokines like IL-6[64] as well as other inflammatory mediators in HCC development. A first attempt to study the potential role of cytokine polymorphisms in determining the risk of HBV-related HCC was made in 2005 by Nieters *et al*[65], who examined the correlation between polymorphisms in Th1 and Th2 cytokine genes in a group of 250 patients with incident HCC and a group of 250 matched hospitalized controls in China: however, none of the study participants presented the C allele of the *IL-6* -174 G/C polymorphism, therefore this polymorphism was not further investigated. Subsequently, a population-based case-control study of HCC, including 120 HCC patients and 230 matched control subjects, was conducted in non-Asian residents of Los Angeles County, California, into genetic polymorphisms in the cytokine genes and risk of HCC. The authors demonstrated that the GG *IL-6* genotype showed the strongest influence on HCC risk among all the cytokine polymorphisms studied[66]. In a more recent study Falleti *et al*[25] investigated whether *IL-6* polymorphisms could be associated with the occurrence of HCC in patients with liver cirrhosis, analyzing 219 consecutive patients who underwent liver transplantation for liver cirrhosis. They found a significant association between the presence of the low-producer genotype (-174 CC) and absence of HCC[67]. Finally, our group performed a study which aimed to evaluate the frequency of SNPs in the *IL-6* promoter region at position -174 and IL-6 serum levels in a group of patients with HCC and underlying liver cirrhosis compared with a group of LC patients without HCC. We found that IL-6 serum levels were higher in G/G compared to C/C genotypes only in HCC; IL-6 serum levels in G carriers were higher in HCC versus LC patients while there were no differences for the C allele. IL-6 serum levels in HCC correlated with G carriers[15] (Table 3).

CONCLUSION

The possibility of a genetic association between -174 G/C polymorphism and some specific liver diseases has been suggested by several studies which are quite unanimous in observing a correlation between the presence of the high-producer genotype (GG) and a worse evolution of the chronic disease. This has been observed in patients with HCV-related chronic hepatitis even with PNALT and in patients with liver cirrhosis and HCC whatever the etiology. Studies on HBV-related chronic hepatitis have not been conclusive because they were performed in populations (generally Asiatic) which have much lower frequencies of the -174 C allele than Caucasian populations. Finally, specific populations like NAFLD/NASH, autoim-

Table 1 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in hepatitis C virus and hepatitis B virus infection

Ref.	Country	Ethnicity	Cases	Controls	Genotyping method	Association with chronic hepatitis/ response to therapy
Barrett *et al*[20]	Ireland	Caucasian	158	-	PCR-SSP	Positive significant/-
Nattermann *et al*[23]	Germany	Caucasian	210	100	Cytokine genotyping tray	-/Uncertain
Falleti *et al*[25]	Italy	Caucasian	121	-	PCR-RFLP	Positive significant /-
Park *et al*[26]	South Korea	Asian	1046	-	PCR-SBE	NS/-
Ribeiro *et al*[27]	Brazil	American[1]	26	41	PCR-SSP	NS/-

[1]White, black and hispanic. PCR-SSP: Polymerase chain reaction - single specific primer; PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; PCR-SBE: Polymerase chain reaction - single base primer extension assay; NS: Not significant.

Table 2 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in alcoholic and autoimmune liver diseases

Ref.	Country	Ethnicity	Cases	Controls	Genotyping method	Association
Carulli *et al*[33]	Italy	Caucasian	79	114	PCR-RFLP	Positive significant (NAFLD)
Fernández-Real *et al*[34]	Spain	Caucasian	32	-	PCR-RFLP	Positive significant (diabetes and lipid abnormalities) (G allele)
Marcos *et al*[48]	Spain	Caucasian	258	101	TaqMan genotyping	NS (alcoholic liver disease)
Fan *et al*[53]	China	Asian	77	-	PCR-RFLP	Positive significant (PBC)

PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; NAFLD: Non-alcoholic fatty liver disease; PBC: Primary biliary cirrhosis.

Table 3 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in hepatocellular carcinoma

Ref.	Country	Ethnicity	Cases	Controls	Genotyping method	Association with HCC
Nieters *et al*[65]	China	Asian	250	250	PCR-RFLP	NS
Ognjanovic *et al*[66]	United States	American[1]	120	230	5'nuclease Taqman allelic discrimination assay	Positive significant
Falleti *et al*[67]	Italy	Caucasian	219	-	PCR-RFLP	Positive significant
Giannitrapani *et al*[15]	Italy	Caucasian	105	-	PCR-RFLP	Positive significant

[1]White, black and hispanic. PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; HCC: Hepatocellular carcinoma; NS: Not significant.

mune and HIV/HCV co-infected patients not achieving SVR showed a higher prevalence of the CC genotype, probably as a result of many other complex immunological, virological and host-related interrelations that cannot be explained by the presence of a unique SNP.

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ORIGINAL ARTICLE

Annexin A1: A new immunohistological marker of cholangiocarcinoma

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Abstract

AIM: To evaluate a new immunohistological marker, annexin A1 (ANXA1), in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC).

METHODS: Expression of ANXA1 protein was investigated in liver tissues from patients with CCA and HCC by immunohistochemistry. Its expression on differences stages of tumor development was investigated in hamster CCA tissues induced by *Opisthorchis viverrini* and *N*-nitrosodimethylamine. Moreover, mRNA expression of ANXA1 was assessed in CCA cell lines by quantitative real-time polymerase chain reaction and silencing of *ANXA1* gene expression using small interfering RNA.

RESULTS: In human CCA tissue arrays, immunohistochemical analysis revealed that the positive expression of ANXA1 was 94.1% (64/68 cases) consisting of a high expression (66.2%, 45/68 cases) and a low expression (33.8%, 23/68 cases). However, expression of ANXA1 protein was negative in all histologic patterns for HCC (46/46 cases) and healthy individuals (6/6 cases). In hamster with opisthorchiasis-associated CCA, the expression of ANXA1 was observed in the cytoplasm of inflammatory cells, bile duct epithelia and tumor cells. Grading scores of ANXA1 expression were significantly increased with tumor progression. In addition, mRNA expression of ANXA1 significantly increased in all of the various CCA cell lines tested compared to an immortalized human cholangiocyte cell line (MMNK1). Suppressing the *ANXA1* gene significantly reduced the matrix metalloproteinase (MMP) 2 and MMP9, and transforming growth factor-β genes, but increased nuclear factor-κB gene expression.

CONCLUSION: ANXA1 is highly expressed in CCA, but low in HCC, suggesting it may serve as a new immunohistochemical marker of CCA. ANXA1 may play a role in opisthorchiasis-associated cholangiocarcinogenesis.

Key words: Cholangiocarcinoma; *Opisthorchis viverrini*; Hepatocellular carcinoma; Annexin A1; Biomarker

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INTRODUCTION

Cholangiocarcinoma (CCA) is the leading cancer cause of death in northeastern Thailand. The incidence of CCA, a bile duct cancer, from the Khon Kaen Cancer Registry, Thailand, between 1985 and 2009 was 44.3 per 100000 in the males and 17.6 per 100000 in the females[1] which was the highest incidence rate in Southeast Asia[2,3], while its incidence is quite low in European countries[4]. The high incidence of CCA is found mainly in persons over 35 years of age and varies from 93.8 to 317.6 per 100000 person-years[5]. The incidence of mainly the intrahepatic type of CCA most typical in the northeastern part of Thailand is associated with the high prevalence of opisthorchiasis caused by *Opisthorchis viverrini* (*O. viverrini*) infection[5,6]. Because CCA is lacking specific symptoms and with no early diagnostic markers, the patients often present at late onset when the disease is in the advanced stage and the patients end up with a poor prognosis[7]. After surgery, no effective drug treatment is available[8] resulting in short survival outcomes[8,9].

The histologic distinction between CCA and hepatocellular carcinoma (HCC) is difficult due to heterogeneity and similarities in morphology[10-13]. Although several immunohistochemical markers such as cytokeratin (CK) 7, CK20, and hepatocyte paraffin 1 (HepPar1) are widely used to distinguish CCA and HCC, these markers can be expressed by both cancers[14,15]. Therefore, novel diagnostic markers for diagnostic differentiation of primary liver tumors are required.

Since the liver fluke associated with intrahepatic CCA is believed to have an immunopathological effect[6,16], a proteomics based approach was used to search for protein alterations during the host-parasite interaction in an *in vitro* study. A candidate molecule namely annexin A1 (ANXA1) was found which significantly upregulated persistently during the long-term host-parasite interaction. ANXA1 has diverse functions including the regulation of cell division, proliferation, apoptosis and cell growth. Its function participates in stimulation of epithelial cell motility which is crucial in the development of metastasis by disruption of cell morphology[17]. To date, however, the expression of ANXA1 in CCA and its relationship with clinicopathologic factors is unclear. Up-regulation[18] and down-regulation[19] expressions of ANXA1 have been found in sporadic CCA. Similar to in HCC, its expression is controversial, both up-regulation[20,21] and down-regulation[22] have been reported.

In the present study, the expression of ANXA1 in tumor tissues of intrahepatic CCA and HCC patients and its relationship with the clinicopathologic factors was investigated by immunohistochemical staining. In addition, opisthorchiasis-associated CCA in hamsters at the different stages of tumor development was assessed. The expression and regulation in CCA cell lines of ANXA1 was also determined *in vitro* using small interfering RNA (siRNA) to suppress the *ANXA1* gene.

MATERIALS AND METHODS

Patient tissue samples

Tissue microarrays (TMAs) were constructed from archival paraffin embedded tissue samples of 68 intrahepatic CCA patients, 46 HCC patients and tissues from 6 normal healthy livers. These patients underwent liver resection at Srinagarind Hospital, Khon Kaen University, Thailand during 1999-2010. Diagnosis of both CCA and HCC patients were evaluated by clinical data, imaging analysis, tumor markers, and pathology. Immunohistochemical studies for pathological diagnosis included antibodies to CK7, cancer antigen or carbohydrate antigen 19-9 (CA19-9), HepPar1 and alpha-fetoprotein (AFP). The tumor tissues were verified based on the following criteria: as CCA when either $CK7^+$ or $HepPar1^-$, with or without $CA19\text{-}9^+$, were found; or as HCC when either $HepPar1^+$ or $CK7^-$, with or without AFP^+, were found. The study protocol was approved by The Human Research Ethics Committee, Khon Kaen University, Thailand (HE551407).

TMA and immunohistochemistry

TMAs of 68 *O. viverrini*-associated CCA cases and 46 HCC cases were generated manually from the paraffin-embedded tissues. In brief, four randomly selected regions from each paraffin block were identified on a hematoxylin-eosin (HE)-stained slide, after which the slide was aligned with the surface of the original paraffin block to locate the sampling areas. The designated areas in the paraffin block were punched with a 1-mm-diameter needle before each punched tissue was then manually transferred to a new recipient paraffin block to generate a TMA block. Five-micrometer-thick sections were cut from the TMA block, and applied on silane-coated slides (Sigma, St. Louis, MO, United States). After TMA construction, to confirm the presence of intact tumor tissue, a HE stained section of the TMA block was prepared and reviewed by two independent pathologists.

An immunohistochemical reaction was performed on 5 μm-thick sections of TMA on silane-coated slides (Sigma) by an immunoperoxidase method. Tissue samples were deparaffinized and dehydrated before the endogenous peroxidase activity was blocked by adding 3% H_2O_2

in phosphate buffer saline (PBS) for 30 min. After washing with PBS, pH 7.4, and blocking with 2% skim milk in PBS, pH 7.4, for 30 min, the samples were incubated with rabbit polyclonal anti-ANXA1 antibody (1:100, Santa Cruz, Heidelberg, Germany) diluted in 2% nonfat dried milk, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:400, GE healthcare, Piscataway, NJ, United States). The appearance of the brown color corresponding to the peroxidase activity was developed using 3,3-diaminobenzidine tetrahydrochloride as a chromogen and counterstained with Mayer's hematoxylin. Negative controls were performed in a similar manner but omitting the primary antibody. The ANXA1 staining was scored based on signal intensity and positive area as follows: negative, < 10%; weak (+), 10%-25%; moderate (++), 26%-75%; and strong (+++), > 75%. Consensus evaluation from at least two of the three investigators was considered acceptable.

Animal tissue samples

The Animal Ethics Committee of Khon Kaen University, Thailand approved the study protocols for the animal experiments (AEKKU 17/2552). Thirty male Syrian golden hamsters (*Mesocricetus auratus*) aged between 4 and 6 wk were obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Animals were divided into two groups; normal control group and infection with *O. viverrini* plus *N*-nitrosodimethylamine (NDMA) (*O. viverrini* + NDMA). In the treated group, hamsters induced by infection with *O. viverrini* metacercariae isolated from naturally infected cyprinid fish by pepsin (Wako, Japan) digestion and subsequently treated with 12.5 ppm NDMA at the same time point as described previously[23]. NDMA was given in drinking water for 2 mo, and withdrawn thereafter until the animals were sacrificed at 21 d, and then at 3 and 6 mo post-treatment (n = 5 for each sub-group). Animals were anaesthetized and killed with an overdose of diethyl ether. The liver was dissected and was placed in 10% buffered formalin and used to evaluate histopathological changes and immunohistochemical studies[23].

Cell lines and cell culture

Four human CCA cell lines, namely M156, M055, M213 and M214 were isolated from intrahepatic CCA patients from northeastern Thailand. CCA tissues were characterized as M156, M055 and M214 moderately differentiated CCA and M213 adenosquamous cell carcinoma. Those CCA cell lines were used to assess *ANXA1* gene expression and compared to an immortalized human cholangiocyte cell line (MMNK1). In addition, the M214 CCA cell line was used to verify ANXA1-related molecule regulation. These CCA cell lines were kindly provided by Associate Professor Banchop Sripa. All cell culture materials (media, serum and antibiotics) were purchased from Gibco, Invitrogen (Auckland, New Zealand). The CCA cells were cultured in HAM's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% Penicillin-streptomycin, 1.176 g/L sodium bicarbonate and adjusted to pH 7.1 with 1 mol/L HCl. The cells were incubated at 37 ℃ under a humidified 5% CO_2 atmosphere.

Western blot analysis

Protein was extracted from the CCA cell lines and the concentration was measured by the Bradford assay (Bio-Rad, Hercules, United States) according to the manufacturer's instructions. Twenty micrograms of protein were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ, United States) for 2 h at 60 V. The membrane was incubated overnight at 4 ℃ with rabbit polyclonal anti-ANXA1 antibody (1:1000, Santa Cruz, Heidelberg, Germany) diluted in 2% nonfat dried milk/phosphate buffered saline with Tween 20 (PBS-T). Subsequently, the membrane was incubated with an appropriate HRP-conjugated secondary antibody (1:3000, GE healthcare) diluted in 2% nonfat dried milk/PBS-T. The immunoreactive materials were developed by enhanced chemiluminescence using the ECL Western blotting Detection Reagent (GE Healthcare).

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA extraction was performed from various CCA cell lines (3×10^5 cells) of each experiment using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States). An aliquot of total RNA was reverse transcribed into cDNA using reverse transcriptase (Invitrogen) following the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out in duplicate in a 20-μL volume using Faststart Universal SYBR Green Master (ROX, Roche Applied Science, Penzberg, Germany) using the following sets of primers: matrix metalloproteinase 2 (MMP2) (5'-TTGATGGCATCGCTCAGATC-3' and 5'-CTGCGAAGAACACAGCCTTC-3'), MMP9 (5'-CATTGTCATCCAGTTTGGT G-3' and 5'-ACCACAACTCGT CGTCGTC-3'), transforming growth factor (TGF)-β (5'-ACATCGACTTTCGCAAGGAC-3' and 5'-TGGTTGTAG AGGGCAAGGAC-3'), nuclear factor-κB (NF-κB) (5'-GCTTTGCAAACCTGGGAATA-3' and 5'-CAAGG TCAGAATGCACCAGA-3'), and GAPDH (5'-AGAAGACTGTGGATGGCCCC-3' and 5'-TGACCTTGCCCACAGCCTT-3'). Reactions were performed in the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, United States). All data were analyzed using 7500 system software with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta Ct}$ method.

Knockdown of ANXA1 using siRNA against the ANXA1 gene

The M214 CCA cells were transiently transfected with siRNA against the *ANXA1* gene (Silencer® Select siRNA; siRNA ID s1380, Ambion, TX, United States) using the lipofectamine™ 2000 transfection reagent (Invitrogen) following the manufacturer's protocol with some modi-

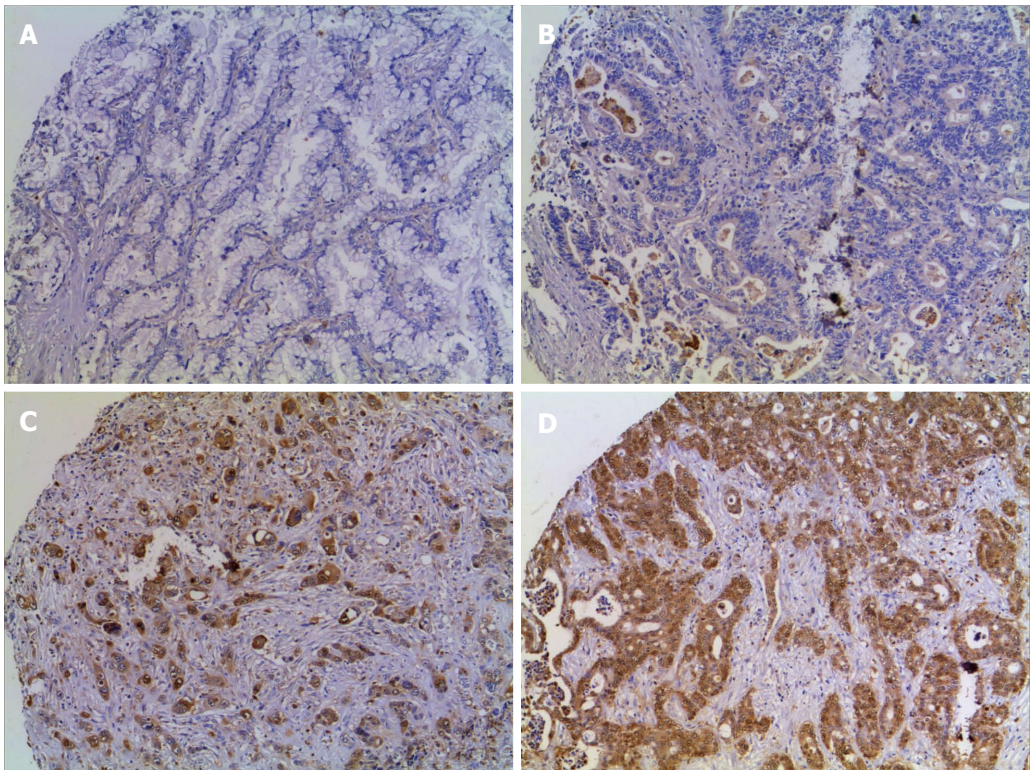

Figure 1 Immunostaining of annexin A1 protein expression in cholangiocarcinoma tissue microarrays. A-D: Tissue microarray of cholangiocarcinoma samples was stained with anti-annexin A1 antibody and counter stained with Mayer's hematoxylin and represented by A for negative, B for +, C for ++, and D for +++ when expression was < 10%, 10%-25%, 26%-75% and > 75%, respectively (magnification, × 200).

fications. In brief, 5 μL of 5 μmol/L stock concentration siRNA or scrambled siRNA and 5 μL lipofectamine™ 2000 transfection reagent were separately diluted in 250 μL OPTI-MEM I medium (Invitrogen). The diluted siRNA solution was mixed with the diluted transfection reagent and incubated for 20 min at room temperature before being added to a six-well plate seeded with 1×10^5 CCA cells in 2 mL transfection medium. The level of ANXA1 mRNA was accessed at 48 and 72 h after transfection. The negative controls were performed by using Silencer® Negative Control siRNA No. 1 (Ambion), a non-targeted sequence.

Statistical analysis

The data were expressed as mean ± SD. To compare the data between groups, statistical significance was determined by Student's *t*-test. The χ^2 test was used to analyze the correlation between ANXA1 expression and the categorical variables regarding clinicopathological parameters. Survival analysis was done by Kaplan-Meier and log-rank tests. Statistical analyses were performed using SPSS version 15 (SPSS, Inc, Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Expression of ANXA1 as a potential diagnostic marker for CCA but not HCC

ANXA1 expression in TMA obtained from 68 CCA and 46 HCC patients was examined using immunohistochemistry. Expression of ANXA1 was observed in the cytoplasm of epithelial bile duct tumor cells and some of inflammatory cells but was seen faintly or with no staining in tumor stroma and in hepatocytes (Figure 1). Sixty-four cases were positive (94.1%, 64/68) for ANXA1 expression. A high expression was coded in 21 cases for ++ and 24 cases for +++ or 66.2% (45/68 cases) and low expression where 4 cases were negative and 19 cases for + or 33.8% (23/68 cases) (Table l; Figure 1). The histological feature in the tubular type showed that high expression (78.8%, 26/33) was significantly higher than low expression (21.2%, 7/33) ($P < 0.05$) (Table 1).

To determine ANXA1 expression distinguishing CCA from HCC, ANXA1 expression was determined in TMA of 46 HCC patients. Slight expression of ANXA1 was observed in the cytoplasm of some inflammatory cells, but was seen faintly or with no staining in tumor cells and hepatocytes. All histologic patterns of HCC samples 100% (46/46) were negative or of low expression (44 for negative and 2 for +) as shown in Figure 2. In addition, no staining or faintly stained immunoreactivity in normal liver tissues of healthy individuals (6/6) was also found. ANXA1 protein as an immunohistological marker could detect CCA and differential CCA from HCC in the primary liver cancer similar to CK7 and CA19-9 (Figure 3). ANXA1 immunohistochemistry showed high sensitivity (94%) and specificity (100%) and the positive prediction value was 100%.

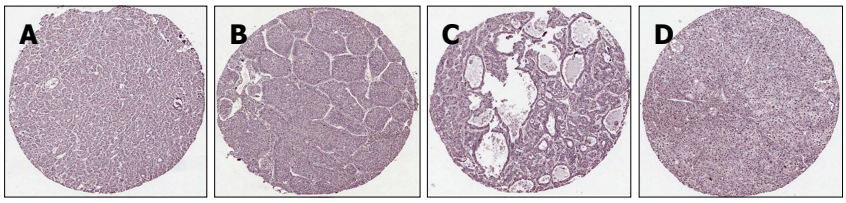

Figure 2 Immunostaining of annexin A1 in hepatocellular carcinoma tissue microarrays. Tissue microarray of hepatocellular carcinoma (HCC) samples was stained with anti-annexin A1 (ANXA1) antibody and counter stained with Mayer's Hematoxylin. ANXA1 shows low expression in all histologic patterns of HCC. A: Trabecular pattern; B: Broad trabecular pattern; C: Trabecular with pseudoacinar pattern; D: Solid growth pattern (magnification, × 40).

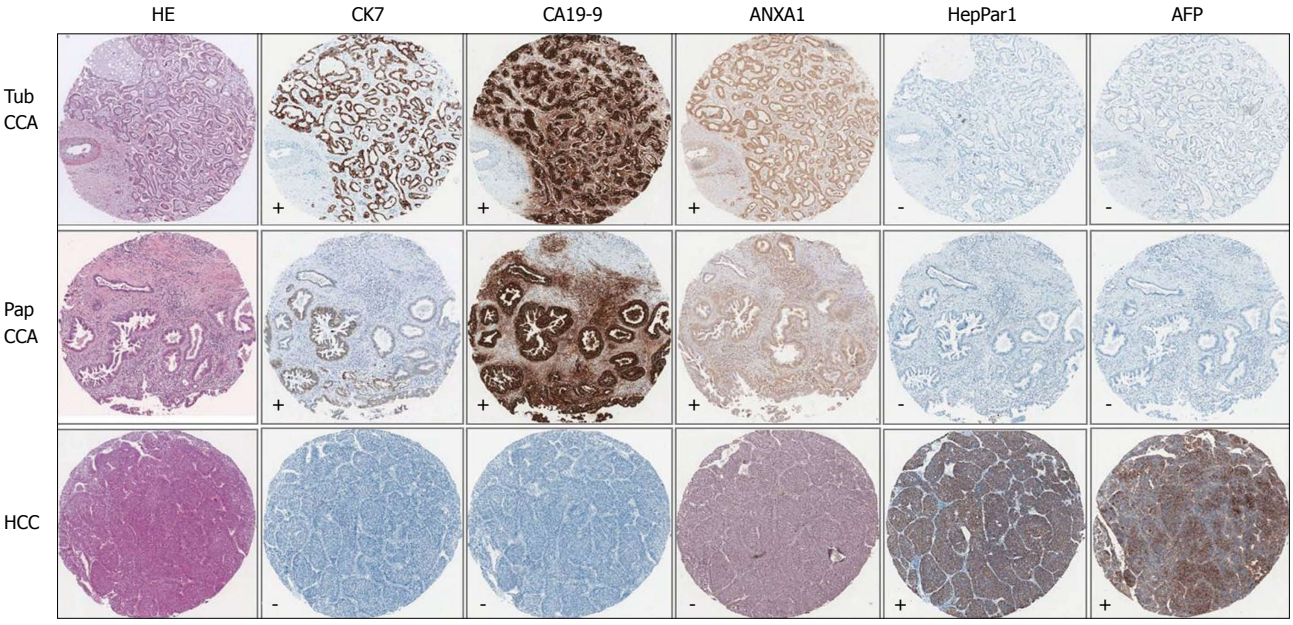

Figure 3 Illustration of the comparative immunohistochemical stains in 3 representative cases. The 3 representative cases include tubular type cholangiocarcinoma (CCA) (Tub CCA), papillary type CCA (Pap CCA) and hepatocellular carcinoma (HCC). Immunopositivity of cytokeratin 7 (CK7), annexin A1 (ANXA1), hepatocyte paraffin 1 (HepPar1) and alpha-fetoprotein (AFP) appeared as brown cytoplasmic staining of tumor cells, while positivity to carbohydrate antigen 19-9 (CA19-9) appeared as cytoplasmic and luminal staining of tumor (magnification, × 40). Noted that CCA are $CK7^{+}$/$CA19\text{-}9^{+}$/$ANXA1^{+}$ and HCC is $HepPar1^{+}$/AFP^{+}/$ANXA1^{-}$. -: Negative result; +: Positive result.

The correlation between ANXA1 expression and clinicopathological parameters was analyzed as shown in Table 1. There were no correlations between ANXA1 expression level and age, sex, tumor location, tumor size, gross type, lymph node metastasis (Table 1) and patients' survival outcome (data not shown). Notably, high ANXA1 expression levels were positively correlated with the histological features of the tubular type ($P = 0.03$) but not the papillary type. All 26 cases of high expression in the tubular type had positive lymph nodes.

Expression of ANXA1 in hamsters CCA tissues

To evaluate the expression of ANAX1 in the different stages of CCA tissues in *O. viverrini* + NDMA-induced CCA in the hamster model, immunohistochemistry was used to evaluate the inflamed tissues at 21 d, when the tumors began at 3 mo, and tumor progression at 6 mo post-treatment. The percentage of CCA 60% (3/5) at 3 mo, and 100% (5/5) at 6 mo post-treatment were described previously[23]. Here, it was shown that the expression of ANXA1 was observed mainly in the cytoplasm of the epithelial bile ducts, some inflammatory cells (large cell, macrophage-like cell), and tumor cells which increased with time as bile duct proliferation and CCA development progressed (Figure 4). The level of its expression at 21 d was +, 3 mo was ++, and 6 mo was +++ for all five animals per group. In addition, low or a little expression was found in the normal livers with no changes in each time-sacrificed group.

Expression of ANXA1 and the effect of its gene inhibition in CCA cell lines

Since ANXA1 was positive in CCA tissue but not in HCC tissue, the expression was further confirmed in various intrahepatic human CCA cell lines compared to MMNK1. Real-time reverse transcription (RT)-PCR revealed that a significantly increased expression of the *ANXA1* gene was observed in all four CCA cell lines including M156, M055, M213 and M214 compared to in MMNK1 ($P < 0.01$) (Figure 5).

In addition, ANXA1 was also investigated whether it was involved in MMPs in CCA metastasis by silenc-

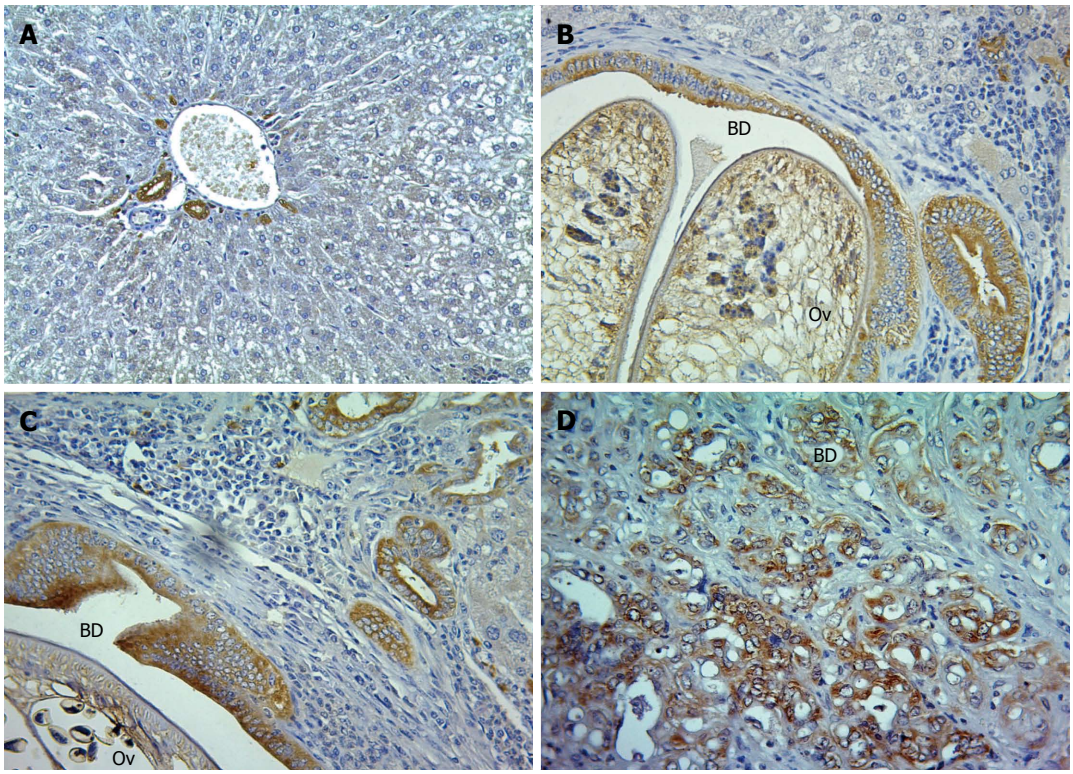

Figure 4 Immunostaining of annexin A1 protein expression in hamster tissues. A-D: Hamster tissues were stained with anti-annexin A1 antibody and counterstained with Mayer's hematoxylin represented by negative for A (normal at 6 mo post-treatment), grade + for B (21 d post-treatment), grade ++ for C (3 mo post-treatment) and grade +++ for D (6 mo post-treatment) (magnification, × 200). Ov: *Opisthorchis viverrini*; BD: Bile duct lumen.

Table 1 Clinical-pathological variables and the expression status of annexin A1 in cholangiocarcinoma tissues

Variables	Annexin A1			*P* value
	Low	High	Total	
Age (yr)				0.328
≤ 56	11	16	27	
> 56	12	29	41	
Gender				0.487
Male	15	33	48	
Female	8	12	20	
Histopathologic feature				0.033
Tubular type	7	26	33	
Papillary type	16	19	35	
The intrahepatic location				0.197
Peripheral	11	16	27	
Hilar	11	29	40	
Gallbladder bed	1	0	1	
Tumor size (cm)				0.889
< 3	5	10	15	
3-6	10	17	27	
> 6	8	18	26	
Gross type				0.244
Mass forming	10	28	38	
Periductal infiltrating	6	9	15	
Intraductal	7	7	14	
Lymph node metastasis				0.382
Absent	7	21	28	
Present	11	20	31	

When the sum of subset numbers does not match patient totals, data were missing or unavailable.

ing ANXA1 using siRNA to target of *ANXA1* gene expression in M214 CCA cells. Real-time RT-PCR and Western blotting analysis showed that the mRNA level of cells transfected with siRNA at 48 h was suppressed by 87.3% and the protein level was decreased by 80% compared to that of controls (Figure 6). *In vitro* proliferation of the ANXA1 silenced cells was also reduced (data not shown). Suppression of the *ANXA1* gene significantly inhibited the mRNA expression of MMP2 by 50% and MMP9 by 45% ($P < 0.05$), and TGF-β by 15%, but induced NF-κB by 70% (Figure 7).

DISCUSSION

The most frequent malignancy among Thais from northeastern Thailand registered between 1985 and 2009 in liver cancers was 42% HCC and 58% CCA[1]. Distinguishing CCA from HCC can be problematic due to heterogeneous morphological. Here, ANAX1, a candidate marker obtained from proteomics was used to investigate CCA in humans and hamsters by immunohistochemistry and tested its regulation expression in an *in vitro* study. In human TMAs of intrahepatic CCA, expression of ANXA1 protein was highly expressed (94.1%, 64/68), similar to a previous study (83.3%, 10/12) in sporadic intrahepatic CCA[18]. An increased expression of the *ANXA1* gene was verified in all of four CCA cell lines, supporting its over-expression in CCA tissue. In contrast, a decreased

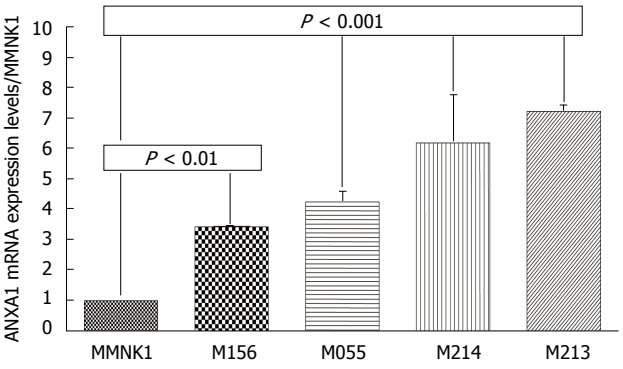

Figure 5 Expression of the annexin A1 gene in various cholangiocarcinoma cell lines. The mRNA expression level of annexin A1 (ANAX1) was evaluated by real-time reverse transcription-polymerase chain reaction in various cholangiocarcinoma (CCA) cell lines including M156, M055 and M214 moderately differentiated CCA, M213 adenosquamous cell carcinoma and compared to an immortalized human cholangiocyte cell line (MMNK1). Data are derived from duplicate independent experiments and presented as mean ± SD, $P < 0.01$, $P < 0.001$ *vs* MMNK1 is significantly different by the Student *t*-test.

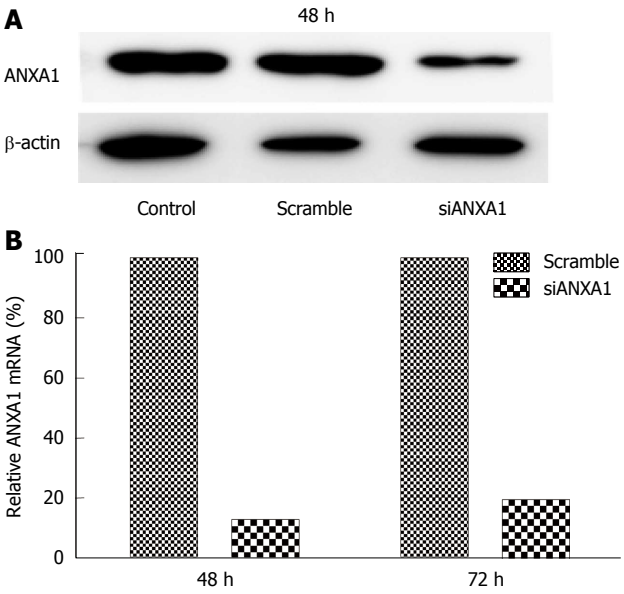

Figure 6 Effect of annexin A1 knockdown on the M214 cholangiocarcinoma cell line. A, B: Suppression of annexin A1 (ANXA1) expression at the translational level (at 48 h) and transcriptional level (at 48 and 72 h) in knockdown M214 cells was evaluated by Western blotting (A) and real-time reverse transcription-polymerase chain reaction (B).

expression of ANXA1 is a common event in extrahepatic CCA and is significantly correlated with a poorer outcome in Chinese patients[19], indicating that its expression is varied according to tumor location. Moreover, down-regulation of ANXA1 expression was found in the HCC tissue array reported by Xue *et al*[22] but opposite from the previous report in transgenic mice with HCC[20] and in a human HCC cell line[21]. Therefore, expression of ANXA1 has different expression in primary liver cancer. With the current results and previous study as evidence, this indicates that ANXA1 is a new immunohistochemical marker to distinguish between CCA and HCC. Nevertheless, more studies in sporadic CCA are warranted.

ANXA1 is bound to cellular membranes in a Ca^{2+}-dependent manner. It is a glucocorticoid-induced protein with multiple actions in the regulation of inflammatory cell activation, cellular processes and involved in carcinogenesis[24]. Recently, its expression has been proposed for the regeneration of skeletal muscle tissue[25] and in liver regeneration and transformation[20]. In hamster CCA, ANXA1 expression has been observed mainly in bile duct epithelia and increasing with bile duct proliferation and bile duct cancer progression in this current study. It may imply that ANXA1 may contribute to regenerate bile duct injury triggered by the fluke and NDMA-mediated CCA. Likewise, in mice after hepatectomy, regeneration of hepatocytes and bile ducts may lead to activate ANXA1 expression in the liver[20].

In addition, although its expression was not positively correlated with patients' survival, and lymph node invasion, most cases of a high expression were found in the tubular type having positive lymph nodes, implying that it may be involved in tumor invasion[26] and specific for the tubular type but not in the papillary type. ANXA1 may regulate MMPs expression for CCA progression. The *in vitro* study revealed that ANXA1 in CCA cell lines of intrahepatic CCA patients was positively correlated with TGF-β and MMP2 and MMP9, but had a negative relationship with NF-κB. ANXA1 inhibits NF-κB, a key regulator of inflammation, the common pathophysiological mechanism of inflammatory bowel diseases[27]. These results indicated that ANXA1 functions as a positive regulator of TGF-β, MMP2 and MMP9 expression and invasion of cancer cells through specific activation of the NF-κB signaling pathway[28]. ANXA1 may regulate TGF-β signaling and promote metastasis[29] leading to up-regulation of MMP2 and MMP9[30] to degrade extracellular matrix (ECM) for tumor development and metastasis. The chronological expression of ANXA1 was shown to have different expression levels according to tumor development. An increased expression of ANXA1 with time is likely to be positively correlated with an increased accumulation of fibrosis and ECM, MMP9 expression[23] for maintenance of cytoskeleton and ECM integrity, and differentiation[25] for tumor onset[31] and tumor progression[32].

Recently, expression of ANXA1 was reported in inflamed tissue such as in ulcerative colitis[33] and in chronic granulomatous inflammation which might become activated at different stages of this chronic inflammatory response[34]. Moreover, expression of ANXA1 was correlated with the tumor staging in adenocarcinoma of the esophagus and the esophagogastric junction[35] and bladder cancer[32]. The results of the current study revealed that expression of the ANXA1 level in the inflamed tissues on 21 d was lower than when the tumor began at 3 mo and lower than in the tumor progression at 6 mo post-treatment in the hamster model, this finding may be supported by previous findings. In contrast, in benign breast tissue, myoepithelial cells showed stronger expression of ANXA1 than in tumorous tissue of breast cancer. A decreased expression of ANXA1 is correlated

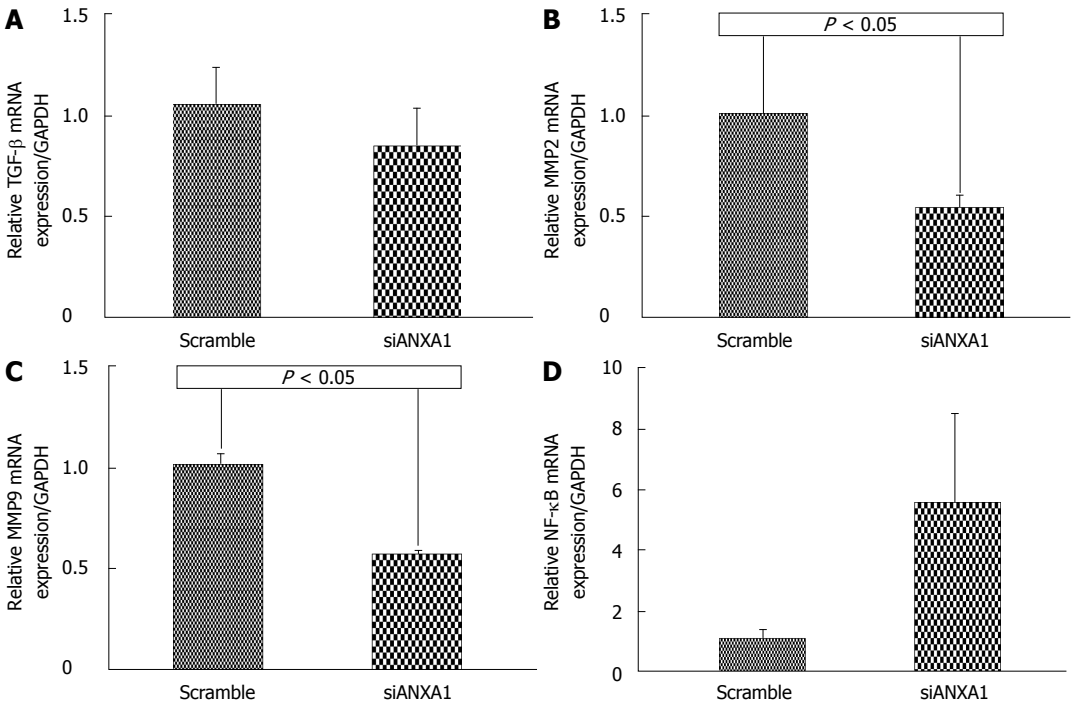

Figure 7 Effect of gene expression in the knockdown of the annexin A1 gene in the M214 cholangiocarcinoma cell line. A: Relative transforming growth factor-β (TGF-β) mRNA expression; B: Relative matrix metalloproteinase (MMP) 2 mRNA expression; C: Relative MMP9 mRNA expression; D: Relative nuclear factor (NF)-κB mRNA expression. Real-time reverse transcription-polymerase chain reaction was used to confirm the expression of TGF-β, MMP2, MMP9 and NF-κB in the knockdown of the annexin A1 (*ANXA1*) gene in the M214 cholangiocarcinoma cell line relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are derived from duplicate independent experiments and presented as mean ± SD, and $P < 0.05$ *vs* scramble is significantly different by the Student *t*-test.

with breast cancer development and progression[36].

In conclusion, strong expression of ANXA1 was observed in CCA, but was low in HCC. In diagnosis of primary liver cancer, ANXA1 could be a new immunohistochemical marker for differential diagnosis of CCA from HCC. ANXA1 expression increased along with cholangiocarcinogenesis in hamsters induced by *O. viverrini* infection and NDMA administration. Its expression was positively correlated with TGF-β and MMPs but negatively correlated with the NF-κB signaling pathway, suggesting that ANXA1 is involved in inflammation-associated CCA, and a potential for therapeutic drug targeting.

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COMMENTS

Background

Cholangiocarcinoma (CCA) is associated with late presentation, has high mortality rate, and poses challenges for diagnostic. The histological CCA is difficult distinguish from hepatocellular carcinoma (HCC). New diagnostic markers with better diagnostic differentiation are required. Annexin A1 (ANXA1) is a multipotent protein involved in several functions including regulation of cell differentiation, apoptosis, and carcinogenesis. ANXA1 involved in this process is frequently overexpressed in many types of cancers. Moreover, its expression in the same cancer is controversial.

Research frontiers

Although the expression of ANXA1 has been demonstrated in many types of cancers, its expression in CCA and HCC is controversial. Moreover, its expression at different stages of CCA development and its regulation in CCA has not been reported.

Innovations and breakthroughs

In this study, strong expression of ANXA1 was observed in CCA, but was low in HCC, which was similar to the available markers for CCA (cytokeratin 7 and carbohydrate antigen 19-9), implying its expression could present as a new marker for differential diagnosis of primary liver cancer. ANXA1 increased expression along with cholangiocarcinogenesis in hamsters induced by *Opisthorchis viverrini* infection and *N*-nitrosodimethylamine administration. Its expression was positively correlated with transforming growth factor-β (TGF-β) and matrix metalloproteinase (MMP) but negatively with the nuclear factor κB (NF-κB) signaling pathway. ANXA1 is involved in carcinogenesis of chronic inflammation related-CCA. ANXA1 is a promising biomarker and a potential for a therapeutic target in this aggressive cancer.

Applications

This study demonstrated that the ANXA1 protein was expressed in CCA, but had low expression in HCC and therefore it may provide a new diagnostic marker in CCA by the immunohistochemistry technique. By silencing of ANXA1 gene expression in the *in vitro* study the results demonstrate that ANXA1 represents a potential therapeutic target for the future intervention in CCA patients.

Terminology

Annexin A1 (also named macrocortin, renocortin, lipomodulin and lipocortin 1) is a 37 kDa protein member of an annexin superfamily which has a binding or annexing property to acidic phospholipid in a calcium dependent manner. It also has been shown to play a critical role in the regulation of inflammatory cell activation, cellular processes and involved in carcinogenesis.

Peer review

This is a good paper with sensible use of different experimental techniques. The authors examined the expression of ANXA1 in CCA and HCC tissues microarrays; ANXA1 protein had high sensitivity and specificity for immunohistochemistry diagnosis in CCA. Suppression of *ANXA1* gene expression showed it inhibiting of TGF-β and MMPs but induction NF-κB expression. The results are interesting, represent a new immunohistochemistry analysis of CCA and ANXA1 is a promising therapeutic potential of drugs targeting in CCA.

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ORIGINAL ARTICLE

Hepatocellular carcinoma in patients with chronic kidney disease

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Abstract

AIM: To investigate outcomes of hepatocellular carcinomas (HCCs) in patients with chronic kidney disease (CKD).

METHODS: Four hundred and forty patients referred between 2000 and 2002 for management of HCCs were categorized according to their CKD stage, *i.e.*, estimated glomerular filtration rate (eGFR) > 90 (stage 1), 60-90 (stage 2), 30-60 (stage 3), 15-30 (stage 4), and < 15 (stage 5) mL/min per 1.73 m^2, respectively. Demographic, clinical and laboratory data were collected and mortality rates and cause of mortality were analyzed. The mortality data were examined with Kaplan-meier method and the significance was tested using a log-rank test. An initial univariate Cox regression analysis was performed to compare the frequency of possible risk factors associated with mortality. To control for possible confounding factors, a multivariate Cox regression analysis (stepwise backward approach) was performed to analyze those factors that were significant in univariate models ($P < 0.05$) and met the assumptions of a proportional hazard model.

RESULTS: Most HCC patients with CKD were elderly, with mean age of diagnosis of 60.6 ± 11.9 years, and mostly male (74.8%). Hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. It was found that patients with stages 4 and 5 CKD were not only older ($P = 0.001$), but also had higher hepatitis C virus carrier rate ($P = 0.001$), lower serum albumin level ($P = 0.001$), lower platelet count ($P = 0.037$), longer prothrombin time ($P = 0.001$) as well as higher proportions of advanced cirrhosis ($P = 0.002$) and HCCs ($P = 0.001$) than patients with stages 1 and 2 CKD. At the end of analysis, 162 (36.9%) patients had died. Kaplan-Meier analysis revealed that patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD (log-rank test, $\chi^2 = 11.764$, $P = 0.003$). In a multivariate Cox-regression model, it was confirmed that CKD stage [odds ratio (OR) = 1.988, 95%CI: 1.012-3.906, $P = 0.046$)], liver cirrhosis stage (OR = 3.571, 95%CI: 1.590-8.000, $P = 0.002$) and serum albumin level (OR = 0.657, 95%CI: 0.491-0.878, $P = 0.005$) were significant predictors for mortality in this population.

CONCLUSION: HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. This warrants further studies.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Chronic kidney disease; End-stage renal disease

Core tip: There is a paucity of data regarding outcomes of hepatocellular carcinoma (HCC) in patients with chronic kidney disease (CKD), even though both hepatitis B virus and CKD are endemic in Taiwan. In a large-scale study, a total of 440 patients with HCC were categorized according to their CKD stage. At the end of analysis, it was found that HCC patients with stages 4 and 5 CKD suffered poorer survival than stages 1 and 2 CKD, which might be explained by inferior liver reserve. Interestingly, tumor stage was not a significant predictor for mortality according to a multivariate Cox regression model.

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INTRODUCTION

Taiwan has the highest prevalence and second highest incidence of end-stage renal disease (ESRD) in the world[1,2]. According to the 2012 Renal Data System of the United States[3], the United States, Taiwan and Japan continue to have some of the highest rates of incident ESRD, at 369, 361 and 288 per million population in 2010. In Taiwan, the prevalence of ESRD reached 2584 per million in 2010, while rates of 2260 and 1870 were reported in Japan and the United States. Similarly, hepatitis B virus (HBV) and hepatocellular carcinoma (HCC) are endemic in Taiwan[4-6]. The carrier rate of hepatitis B surface antigen (HBsAg) in the general population is 15%-20%[4]. Consequently, many Taiwanese patients with CKD or ESRD are also chronic HBV carriers[7].

There is strong evidence of increased cancer risk in patients with CKD[8], in patients with ESRD being treated with chronic dialysis[9-11], and in recipients of renal transplant[12]. The CKD was associated with malignancy and worse prognosis[13-16]. Moderately reduced kidney function may be an independent risk factor for cancer in older men. The risk begins when estimated glomerular filtration rate (eGFR) falls to approximately 55 mL/min and progressively increases as eGFR declines to 40 mL/min, similar to the risk of patients on dialysis or of transplant recipients[8]. Because survival time after the confirmation of recurrence was significantly shorter in the CKD group, their cumulative survival rate was significantly lower compared to that in the non-CKD group, although the difference in disease-free survival was not significant[17].

Almost all cancers in the renal parenchyma are renal cell carcinoma, whereas cancers in the urinary tract are urothelial carcinoma. These two cancers differ markedly in terms of carcinogenesis and basic biology. Renal cell carcinoma is the most common urologic cancer in Western patients on dialysis, whereas urothelial carcinoma is the most common urologic cancer in Asian patients on chronic dialysis[11]. This is a unique and distinguishing epidemiologic characteristic of the Taiwanese population[11]. On the other hand, very few data[17-22] are available regarding the outcome of patients with HCC and CKD, even though both are endemic in Taiwan.

Therefore, this study analyzed the registry of the Chang Gung Memorial Hospital to examine the epidemiology of HCC in CKD populations in Taiwan.

MATERIALS AND METHODS

This retrospective observational study complied with the guidelines of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Chang Gung Memorial Hospital, a tertiary referral center in northern Taiwan. Because of the retrospective nature of this study, Institutional Review Board approval was obtained and the informed consent of risk of HCC and all treatment modalities of all patients on their initial admission was used. Moreover, all individual information was securely protected (by delinking identifying information from the main dataset) and available only to the investigators. All the data were analyzed anonymously and all primary data were collected according to epidemiologic guidelines. This policy was based on previous publications[23,24].

Patients

Four hundred and forty patients referred between 2000 and 2002 for management of HCCs were categorized according to their CKD stage, *i.e.*, eGFR > 90 (stage 1), 60-90 (stage 2), 30-60 (stage 3), 15-30 (stage 4), and < 15 (stage 5) mL/min per 1.73 m^2, respectively. Demographic, clinical, laboratory and mortality data were obtained for analysis.

Diagnosis of HCC

HCC was diagnosed by alpha-fetoprotein, imaging studies such as ultrasonography, radio-contrast enhanced triphasic dynamic computed tomography, magnetic resonance imaging, angiography, and/or documented tissue histopathology[25].

Diagnosis of liver cirrhosis

Cirrhosis was diagnosed by histopathology or by laboratory tests, hepatic ultrasonography, and clinical manifestations of chronic hepatitis with portal hypertension (*i.e.*, varices, thrombocytopenia, or splenomegaly), and/or hepatic decompensation (*i.e.*, jaundice, prolonged prothrombin time, and ascites)[26].

Diagnosis of CKD

The eGFR was computed using the four-variable modification of diet in renal disease (MDRD) study equation[27]. The CKD stage was defined as 1, 2, 3, 4 and 5 according to eGFR; > 90, 60-90, 30-60, 15-30, and < 15 mL/min per 1.73 m^2, respectively[27].

Table 1 Baseline characteristics of patients with hepatocellular carcinoma, stratified according to the stage of chronic kidney disease *n* (%)

Variable	Total (*n* = 440)	Stages 1 and 2 (*n* = 132)	Stage 3 (*n* = 263)	Stages 4 and 5 (*n* = 45)	*P* value
eGFR[1]	53.7 ± 27.1	73.5 ± 13.5	50.0 ± 26.4	17.6 ± 8.7	0.000
Age[1] (yr)	60.6 ± 11.9	54.4 ± 12.3	63.1 ± 10.5	64.0 ± 11.7	0.000
Male	329 (74.8)	115 (87.1)	182 (69.2)	32 (71.1)	0.000
HBV carrier	271 (61.6)	97 (73.5)	152 (57.8)	22 (48.9)	0.002
HCV carrier	201 (45.7)	42 (31.8)	131 (49.8)	28 (62.2)	0.000
HBV and HCV carrier	62 (14.1)	15 (11.4)	39 (14.8)	8 (17.8)	0.488
Follow-up duration[1] (mo)	37.6 ± 37.1	37.0 ± 37.6	40.7 ± 37.6	20.9 ± 27.4	0.013

[1]Data are presented as mean ± SD. The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2. eGFR: Estimated glomerular filtration rate; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Liver function tests of patients with hepatocellular carcinoma, stratified according to the stage of chronic kidney disease (mean ± SD)

Variable	Total (*n* = 440)	Stage 1 and 2 (*n* = 132)	Stage 3 (*n* = 263)	Stages 4 and 5 (*n* = 45)	*P* value
AFP (ng/mL)	6202.1 ± 83392.3	3564.6 ± 11906.0	8176.6 ± 106579.9	1866.3 ± 4866.0	0.911
AST (U/L)	84.8 ± 77.9	94.3 ± 76.1	77.4 ± 68.6	104.3 ± 126.5	0.495
ALT (U/L)	73.0 ± 90.4	79.4 ± 79.1	64.8 ± 49.2	103.0 ± 218.9	0.150
T-bilirubin (mg/dL)	1.8 ± 3.7	1.5 ± 1.6	1.8 ± 4.3	2.8 ± 4.4	0.198
ALP (U/L)	122.4 ± 92.1	124.0 ± 76.6	114.6 ± 79.7	164.5 ± 170.2	0.072
Albumin (g/dL)	3.5 ± 0.7	3.6 ± 0.6	3.5 ± 0.7	3.2 ± 0.7	0.001
Prolonged PT (s)	1.9 ± 4.3	1.4 ± 1.2	1.6 ± 2.1	5.1 ± 12.4	0.000
Platelet count (x 10^3/μL)	138.2 ± 90.7	163.7 ± 109.7	126.5 ± 80.1	131.4 ± 73.0	0.037

The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2. AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T-bilirubin: Total bilirubin; ALP: Alkaline phosphatase; PT: Prothrombin time.

Management of HCC

The HCC patients in the stages 1 and 2 groups underwent tumor resection, liver transplantation, or percutaneous interventional therapies for local tumor ablation, including radio-frequency ablation, pure ethanol injection therapy, pure acetic acid injection therapy, and trans-catheter arterial chemo-embolization[28]. Patients in the stage 3 group received trans-catheter arterial chemo-embolization and radio-frequency ablation[28]. Those in stage 4 received palliative chemotherapy, trans-catheter arterial chemo-embolization, or radiotherapy, and medical care[28].

Statistical analysis

Continuous variables are expressed as means and standard deviations and categorical variables as numbers with percentages in brackets. All data were tested for normality of distribution and equality of standard deviations before analysis. For comparisons between patient groups, we used General Linear Model (Least Significant Difference test) for quantitative variables and Cross-tabulation (χ^2 or Fisher's exact tests) for categorical variables. Mortality data were compared using the Kaplan-Meier method and the significance was tested using a log-rank test. An initial univariate Cox regression analysis was performed to compare the frequency of possible risk factors associated with mortality. To control for possible confounding factors, a multivariate Cox regression analysis (stepwise backward approach) was performed to analyze those factors that were significant in univariate models ($P < 0.05$) and met the assumptions of a proportional hazard model. We considered results that rejected the null hypothesis with 95% confidence to be significant. All analyses were performed using IBM SPSS Statistics Version 20.

RESULTS

Baseline characteristics

Most HCC patients with CKD were elderly, with mean age of diagnosis of 60.6 ± 11.9 years (Table 1), and mostly male (74.8%). Hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. It was found that patients with stages 4 and 5 CKD were older ($P = 0.001$) and had higher hepatitis C virus (HCV) carrier rates ($P = 0.001$) than patients with stages 1 and 2 CKD. On the other hand, there were more male ($P = 0.000$) and HBV carrier rate ($P = 0.002$) in stages 1 and 2 than stages 4 and 5 CKD.

Comparison of liver biochemistry test

Patients with stages 4 and 5 CKD not only had lower serum albumin level ($P = 0.001$) and platelet count ($P = 0.037$), but also had longer prothrombin time ($P = 0.001$) than stages 1 and 2 CKD (Table 2).

Comparison of liver reserve

Patients with stages 4 and 5 CKD had higher incidences of advanced cirrhosis than stages 1 and 2 CKD (Table 3, $P = 0.002$).

Table 3 Liver cirrhosis classification, tumor staging, cause of mortality of patients with hepatocellular carcinoma stratified according to the stage of chronic kidney disease *n* (%)

Variable	Total (*n* = 440)	Stage 1 and 2 (*n* = 132)	Stage 3 (*n* = 263)	Stages 4 and 5 (*n* = 45)	*P* value
Liver cirrhosis classification (Child-Pugh score)					0.002
No	53 (12.0)	27 (20.5)	23 (8.7)	3 (6.7)	
Class A	236 (53.6)	67 (50.8)	151 (57.4)	18 (40.0)	
Class B	110 (25.0)	30 (22.7)	64 (24.3)	16 (35.6)	
Class C	41 (9.3)	8 (6.1)	25 (9.5)	8 (17.8)	
Tumor stage					0.001
Stage 1	213 (48.4)	60 (45.5)	133 (50.6)	20 (44.4)	
Stage 2	113 (25.7)	27 (20.5)	76 (28.9)	10 (22.2)	
Stage 3	84 (19.1)	40 (30.3)	36 (13.7)	8 (17.8)	
Stage 4	30 (6.8)	5 (3.8)	18 (6.8)	7 (15.6)	
Mortality	162 (36.9)	47 (35.6)	94 (35.9)	21 (46.7)	
Cause of mortality					0.050
Liver failure	27 (6.1)	10 (7.6)	16 (6.1)	1 (2.2)	
Tumor death	35 (8.0)	8 (6.1)	22 (8.4)	5 (11.1)	
Gastrointestinal bleeding	27 (6.1)	11 (8.3)	16 (6.1)	0 (0)	
Sepsis	69 (15.7)	18 (13.6)	36 (13.7)	15 (33.3)	
Others	5 (1.1)	1 (0.8)	4 (1.5)	0 (0)	

The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2.

Table 4 Cox regression analysis of mortality in patients with hepatocellular carcinoma and chronic kidney disease

Variable	Univariate analysis		Multivariate analysis	
	Odds ratio (95%CI)	*P* value	Odds ratio (95%CI)	*P* value
Chronic kidney disease stage	2.237 (1.376-3.636)	0.001	1.988 (1.012-3.906)	0.046
Liver cirrhosis stage	4.566 (2.331-8.929)	0.000	3.571 (1.590-8.000)	0.002
Tumor stage	3.509 (1.789-6.849)	0.000	2.169 (0.997-4.717)	0.051
Albumin, each increase of 1 mg/dL	0.503 (0.399-0.633)	0.000	0.657 (0.491-0.878)	0.005
Prolonged prothrombin time, each increase of 1 s	1.062 (1.036-1.089)	0.000	0.977 (0.942-1.013)	0.215

Comparison of tumor staging

Most of the HCCs were diagnosed in the early stages (Table 3). Patients with stages 4 and 5 CKD had higher proportions of advanced HCCs than stages 1 and 2 CKD ($P = 0.001$).

Comparison of mortality

At the end of analysis, 162 (36.9%) patients had died (Table 3). It was revealed that patients with stages 4 and 5 CKD suffered higher fatal septic complications than stages 1 and 2 CKD ($P = 0.050$). The one-, three-, and five-year survival rates were 86.2%, 71.3% and 55.9%, respectively (Figure 1). In addition, patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD (Figure 2, log-rank test, $\chi^2 = 11.764$, $P = 0.003$).

Mortality analysis

In a multivariate Cox regression model (Table 4), it was confirmed that CKD stage [odds ratio (OR) = 1.988, 95%CI: 1.012-3.906, $P = 0.046$], liver cirrhosis stage (OR = 3.571, 95%CI: 1.590-8.000, $P = 0.002$) and serum albumin level (OR = 0.657, 95%CI: 0.491-0.878, $P = 0.005$) were significant predictors for mortality.

DISCUSSION

The analytical data demonstrated that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. The reason is unclear but inferior liver reserve in this subgroup should be considered. In a large-scale study in Taiwan[16], there was a higher risk for overall cancer mortality in CKD patients compared to non-CKD patients (adjusted hazard ratio 1.2). Moreover, CKD was associated with increased mortality from liver, kidney, and urinary tract cancers, with adjusted hazard ratios of 1.74, 3.3, and 7.3, respectively. Most importantly, patients with stage 4 CKD had poorer prognosis than the other groups[16], but the reason was also unclear.

In the present study, patients with HCC were stratified according to the stage of CKD. Kaplan-Meier analysis revealed that patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD ($P = 0.003$). A multivariate Cox regression model confirmed that CKD stage ($P = 0.046$), liver cirrhosis stage ($P = 0.002$) and serum albumin level ($P = 0.005$) were significant predictors of mortality. Tumor stage was a significant predictor for mortality in the univariate model ($P = 0.000$), but not after multivariate analysis ($P = 0.051$).

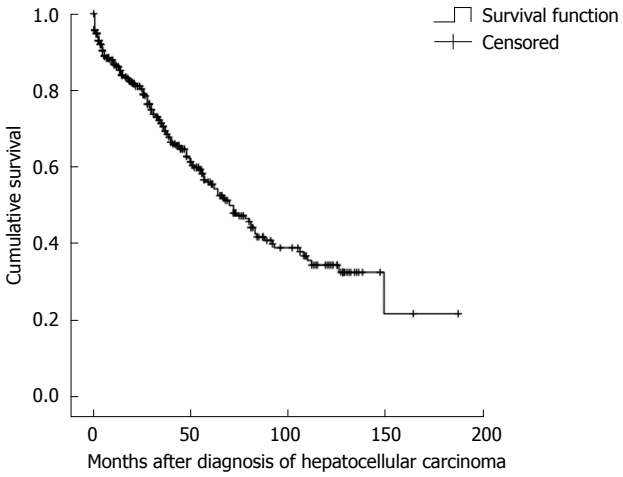

Figure 1 Kaplan-Meier survival analysis. The one-, three-, and five-year survival rate was 86.2%, 71.3% and 55.9%, respectively.

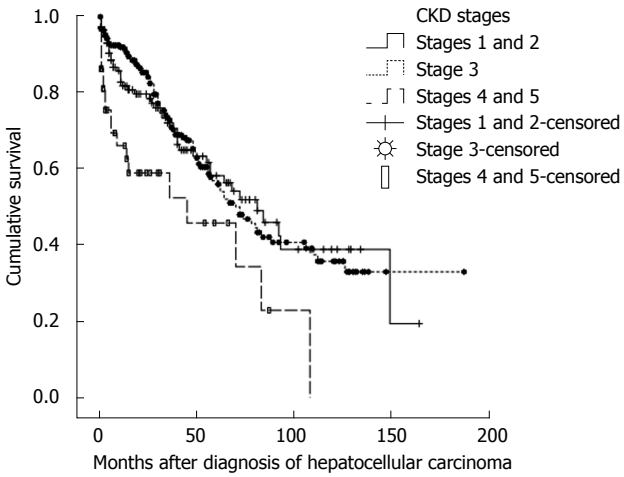

Figure 2 Survival analysis after stratification according to the stage of chronic kidney disease. Patients with stages 4 and 5 chronic kidney disease (CKD) suffered lower cumulative survival than stages 1 and 2 CKD (log-rank test, $\chi^2 = 11.764$, $P = 0.003$).

Previous study[29] also reported that eGFR, as determined by MDRD equation, might provide better prognostic accuracy than the CKD-epidemiology collaboration equations independent of liver functional reserve and tumor staging, and is a more feasible renal surrogate for outcome prediction in patients with HCC and CKD stages 1-3 receiving TACE. Notably, the TNM classification did not accurately predict survival in HCC patients with CKD.

In this study, most HCC patients with CKD are male (74.8%). Moreover, there were more male in stages 1 and 2 than stages 4 and 5 CKD ($P = 0.000$). In 1981, Zevin *et al*[30] reported a hemodialysis patient who developed HCC after long-term therapy with androgenic anabolic steroids. The tumor progressed very rapidly, with no evidence of regression despite discontinuation of the drug. The increased risk of malignancy in patients with chronic uremia and hemodialysis and the higher frequency of HCC associated with the use of anabolic steroids may explain the male predominance in CKD populations[30]. Since CKD is associated with hypogonadism, the protective effect of estrogen on liver cancer may be reduced[16]. Nevertheless, male predominance in HCC has been reported due to other than androgenic anabolic steroid use[31].

In this study, hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. Notably, there were more HCV ($P = 0.001$) in stages 4 and 5 CKD than stages 1 and 2 CKD, but more HBV ($P = 0.002$) in stages 1 and 2 than stages 4 and 5 CKD. Hence, the incidence of HBV (61.6%) is only a little higher than that of HCV (45.7%) in this study, although the national prevalence rates among adults in Taiwan is 1%-3% and 15%-20% for HCV and HBV, respectively[32]. Notably, after implementation of national hepatitis B vaccination in Taiwan, the prevalence of HBsAg among persons younger than 15 years of age has decreased from 9.8% in 1984 to 0.7% in 1999[33]. Higher incidences of HBV and HCV infection were noted in uremic patients, possibly because of cross-infection during hemodialysis. It was found that compared to those who were negative for both markers, patients with both HBsAg and anti-HCV had increased incidence of chronicity[34]. Earlier study[35] also found that HCV infection, but not HBV infection, is significantly associated with prevalence and disease severity of CKD in chronic dialysis patients. However, another population-based study[36] found no association between HCV and risk of development of CKD.

Very few data are available regarding the outcome of HCC in CKD populations. Nevertheless, previous evidences[17-22] suggested that we should be more aggressive on the principle for management for HCC in CKD patients, because the prognosis was not different between patients with and without CKD. For example, Huo *et al*[18] compared the survival of 172 HCC patients with and without CKD who underwent percutaneous injection therapy. After a mean follow-up period of 24 ± 9 mo, there was no significant survival difference in patients with and without CKD [18]. Kondo *et al*[19] also reported that radiofrequency ablation was a safe and effective option for small HCCs in ESRD patients undergoing chronic hemodialysis. Orii *et al*[17] compared the outcomes of 17 patients with CKD who had undergone hepatectomy for HCC with 51 non-CKD patients subjected to hepatectomy for HCC. The operative and pathologic findings were comparable between the two groups. Post-operative circulatory insufficiency occurred more frequently in the CKD group ($P = 0.013$). Although the disease-free survival rates were comparable between the two groups, the overall survival rates were significantly lower in the CKD group than in the non-CKD group ($P = 0.031$). Orii *et al*[17] therefore concluded that hepatectomy for HCC should be considered even for CKD patients if careful peri-operative management and suitable multi-disciplinary treatment for recurrent disease are provided. In the study by Yeh *et al*[20], the outcomes of 26 ESRD-HCC patients who underwent hepatic resection were reviewed and compared to 1198

HCC patients without ESRD who underwent hepatic resection. There were more associated disease, more physical signs of anemia and post-operative complications, lower hemoglobin, platelet, and alpha-fetoprotein levels, elevated blood urea nitrogen and creatinine levels, smaller tumors, lower HBsAg positivity, higher HCV positivity, and longer hospital stays in the ESRD-HCC group compared to the HCC group. Nonetheless, the overall and disease-free survival rates were similar between the two groups[20]. Cheng *et al*[21] also demonstrated that the operative morbidity and mortality between ESRD and non-ESRD groups were similar. The five-year disease-free survival rates for ESRD and non-ESRD groups were 35.0% and 34.2%, respectively ($P = 0.31$), while the five-year actual survival rates were 67.8% and 53.3%, respectively ($P = 0.54$). The study suggested that liver resection for HCC was justified in selected patients with ESRD [21]. In the study by Sawada *et al*[22], 91 patients who underwent hepatectomy were retrospectively divided into two groups based on their creatinine clearance (Ccr) values: a group with Ccr values $\geqslant 50$ to < 100 mL/min ($n = 77$) and a group with Ccr values of $\geqslant 20$ but < 50 mL/min ($n = 14$). There were no statistically significant differences between the two groups in terms of intra-operative blood loss or intra-operative urine volume. The difference between the two groups in post-operative complications was not statistically significant. Thus, the team concluded that adequate indications, appropriate operative procedures, and peri-operative management might allow hepatectomy to be performed safely in patients with non-uremic minimal renal failure[22].

In conclusion, our results showed that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD, which might be explained by poorer liver reserve. Nevertheless, the retrospective nature of the study, the small patient population, and the short follow-up duration are limitations that warrant further investigations to validate the conclusion drawn here.

COMMENTS

Background

There is a paucity of data regarding outcomes of hepatocellular carcinomas (HCCs) in patients with chronic kidney disease (CKD), even though both hepatitis B virus and CKD are endemic in Taiwan.

Research frontiers

Previous reports found a strong evidence of increased cancer risk in patients with CKD, and the CKD was associated with malignancy and worse prognosis.

Innovations and breakthroughs

The authors analyzed the database of Chang Gung Memorial Hospital to examine the epidemiology of HCC in CKD populations in Taiwan. It was found that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. On the other hand, the TNM classification (or tumor stage) did not accurately predict survival in HCC patients with CKD.

Applications

The data is important to understand the outcome of HCC in CKD population in Taiwan, an area with highest prevalence and second highest incidence of end-stage renal disease in the world.

Terminology

HCC is the most common type of liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection or cirrhosis. CKD is the slow loss of kidney function over time. There are five stages of CKD, but kidney function is normal in stage 1, and minimally reduced in stage 2, moderately reduced in stage 3, severely reduced in stage 4, and very severely reduced (or called end-stage renal disease) in stage 5.

Peer review

The epidemiology of HCC in CKD population has not been extensively investigated. Therefore, this data is particularly important.

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ORIGINAL ARTICLE

Overexpression of carbonic anhydrase Ⅱ and Ki-67 proteins in prognosis of gastrointestinal stromal tumors

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Abstract

AIM: To investigate the expression and prognostic value of carbonic anhydrase Ⅱ (CA Ⅱ) and Ki-67 in gastrointestinal stromal tumors (GISTs).

METHODS: One hundred and thirteen GIST patients admitted to Chinese People's Liberation Army General Hospital from January 2004 to December 2010 were retrospectively followed up, and immunohistochemistry was used to detect CA Ⅱ, Ki-67 and CD117 expression in tumor samples. The survival rates of the patients were analyzed using the Kaplan-Meier method. Log-rank test, χ^2 test and Cox proportional hazards model were used to determine the relationships between CA Ⅱ, Ki-67 and CD117 expression and prognostic value in GISTs.

RESULTS: The survival rates at 1, 3 and 5 years were 90.0%, 82.0% and 72.0% in all patients. However, in patients with positive CA Ⅱ or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Compared with the negative groups, the survival rates in the positive groups were significantly lower (CA Ⅱ log-rank P = 0.000; Ki-67 log-rank P = 0.004). Multivariate Cox analysis revealed that CA Ⅱ, CD117 and Ki-67 were considerable immune factors in prognosis of GIST patients (CA Ⅱ P = 0.043; CD117 P = 0.042; Ki-67 P = 0.007). Besides, tumor diameter, mitotic rate, tumor site, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors. Our study indicated that CA Ⅱ had strong expression in GISTs and the prognosis of GISTs with high CA Ⅱ expression was better than that of GISTs with low or no expression, suggesting that CA Ⅱ is both a diagnostic and prognostic biomarker for GIST.

CONCLUSION: CA Ⅱ and Ki-67 are significant prognostic factors for GISTs. CA Ⅱ associated with neovascular endothelia could serve as a potential target for cancer therapy.

Key words: Gastrointestinal stromal tumors; Carbonic anhydrase; CD117; Ki-67; Prognostic factor

Core tip: Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors with a wide spectrum of clinical behavior. This is the first study showing the prognostic significance of carbonic anhydrase Ⅱ (CA Ⅱ) and Ki-67. The 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0%. However, in patients with positive CA Ⅱ or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Our study indicates that CA Ⅱ has strong expression in GISTs and prognosis with high CA Ⅱ expression is better than that with low or no expression, suggesting that CA Ⅱ is both a diagnostic and prognostic biomarker for GIST.

Liu LC, Xu WT, Wu X, Zhao P, Lv YL, Chen L. Overexpression of carbonic anhydrase Ⅱ and Ki-67 proteins in prognosis

of gastrointestinal stromal tumors. *World J Gastroenterol* 2013; 19(16): 2473-2480 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i16/2473.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i16.2473

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract. They show differentiation towards the interstitial cells of Cajal and account for < 1% of all gastrointestinal neoplasms[1]. GISTs positively express discovered on GIST-1 (98%) and CD117 (95%) immune globulin. The estimated incidence of GISTs is 10-20 per million people annually worldwide. The majority of GISTs arise in the stomach (60%), small bowel (30%), esophagus and rectum (10%)[2] and the remainder outside the gastrointestinal tract, comprising a wide spectrum from a curable disorder to highly malignant disease. As far as the molecular markers are concerned, previous studies have revealed that p53, CD147, monocarboxylate transporter 1 (MCT1), DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39) and natural killer cell p30 (NKp30) are related to the prognosis of GISTs[3-7]. However, considering that these markers are different from tumor size, mitotic rate or tumor site, and due to their weak correlation, they are often mentioned in recurrence risk of GIST or prediction of patient prognosis.

Carbonic anhydrases (CAs) are a group of zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide, $CO_2 + H_2O <=> HCO_3^- + H^+$ and participate in various physiological processes, including respiration, gluconeogenesis, bone resorption, renal acidification, and formation of cerebrospinal fluid and gastric acid[8]. To date, 15 isoforms of human (h) α-CA, 15 enzymatically active α CAs have been identified and characterized in mammals, including five cytoplasmic (CA Ⅰ, CA Ⅱ, CA Ⅲ, CA Ⅶ and CA XIII), two mitochondrial (CA VA and CA VB), one secreted (CA Ⅵ), four membrane-associated (CA Ⅳ, CA Ⅸ, CA Ⅻ, and CA XIV) forms and three CA-related proteins (CA-RP Ⅷ, CA-RP X and CA-RP XI). These functionally active CA isozymes, having been identified in mammals[9-11], differ in their tissue distribution and enzymatic activity. Furthermore, in the present study, GIST cells demonstrated strong expression of CA-RPs Ⅷ and XI. Overexpressed CA-RP XI is possibly substituted for CA-RP Ⅷ in the cytoplasm and enhances the proliferation and invasion of GIST cells[12]. These enzymes are commonly expressed in malignant tumor cells in which they promote tumor growth by contributing to intracellular alkalization and extracellular acidification[13]. However, because of its wide distribution, high catalytic efficiency, and an important physiological role, CA Ⅱ has become one of the hot research topics. CA Ⅱ expression in the cytoplasm is a single polypeptide chain of molecular weight 29 kDa and its gene is located on chromosome 8, 8q22, 760 bp. It is present in most tissues with high enzyme activity, including gastric cancer, liver and bile duct cancer, colon cancer, renal cell carcinoma, melanoma, brain astrocyte tumors, pancreatitis cells in mice[14], and cardiomyocyte hypertrophy[15]. In a recent study, high CA Ⅱ expression was associated with a better disease-specific survival rate than low or no expression in GIST[16]. Although CA Ⅱ has been reported to represent potential diagnostic and therapeutic targets in the above cancers, there have been fewer reports discussing the predictive value of prognosis in GIST patients in Asia from a clinicopathological aspect.

Ki-67 is a nuclear marker that is closely related to tumor cell proliferation. It has been found to have a positive correlation with prognosis of various malignant tumors including GIST. One recent study has suggested that Ki-67 is a strong prognostic indicator even though it is less valuable than mitotic rate in GIST[17]. A study by Nakamura *et al*[18] supported the hypothesis that Ki-67 and risk grade are useful for predicting the aggressive biological behavior of GIST.

The aim of our study was to reveal the relationship between the above two molecular markers (CA Ⅱ and Ki-67) and prognosis of GIST. As a diagnostic index, CD117 is located in the tumor cell membrane and cytoplasm[19] and has a positive rate as high as 95% in GISTs. The predictive value of CD117 in prognosis was also explored.

MATERIALS AND METHODS

Study population and follow-up

We retrospectively followed up GIST patients who were admitted and operated upon in Chinese People's Liberation Army General Hospital between January 2004 and December 2010. Clinical follow-up was completed in February 2011. Inclusion criteria were: (1) age ⩾ 18 years; (2) GISTs diagnosed by the histopathological and immunohistochemical methods; and (3) not receiving any previous treatment. Exclusion criteria were: (1) female patients with pregnancy or lactation; (2) patients developing other malignancies during the past 5 years; and (3) patients with other serious diseases.

Pathological examination of tumor samples

Paraffin wax sections (5 μm thick) of GIST specimens were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol and the sections were subjected to heat-induced antigen retrieval using a microwave oven. Sections were incubated overnight at 4 ℃ with polyclonal antibodies for CA Ⅱ, CD117 and Ki-67 (CD117, rabbit anti-human polyclonal antibody, 1:100, Abcam, Cambridge, United Kingdom; Ki67, rabbit polyclonal antibody to proliferation marker, 1:1000, Abcam; CA Ⅱ, rabbit anti-human polyclonal antibody, 1:100, Abcam). Polyperoxidase-anti-mouse/rabbit immunoglobulin G was applied to the sections for 30 min at 37 ℃, followed by

detection with 3,3'-diaminobenzidine (Bioss, Beijing, China). The reactions were developed with hematoxylin and were mounted with glue[20,21]. The immunohistochemical reactions were visualized under high-power magnification (× 400) using an Olympus BH2 microscope (Tokyo, Japan). Positive expression for CA Ⅱ, Ki-67 and CD117 was defined by the percentage of positively stained cells (1 positive; 0 negative). The following scoring assessments for CA Ⅱ, Ki-67 and CD117 were used. Score 0 was assigned for ≤ 10%, and 1 for > 10% staining positive cells.

Ethics

Approval for the use of clinical material for research was obtained from the hospital ethics committee, along with patient consent.

Statistical analysis

SPSS version 17.0 (SPSS Inc. Chicago, IL, United States) was used for statistical analysis. Analysis was performed assuming a nonparametric distribution using the χ^2 test. Actuarial survival rates were evaluated by Kaplan-Meier analysis and log-rank test. Multivariate survival analysis was performed by Cox proportional hazards model. All tests were two-tailed and statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics

A total of 113 GIST patients (61 male, 52 female) with a median age of 60 years were included. Twenty-five patients died from GIST. Median follow-up time was 35.5 mo (1-90 mo).

Expression of CA Ⅱ, Ki-67 and CD117 in tumor samples

Immunohistochemistry showed that the positive rate for CA Ⅱ, CD117 and Ki-67 was 87.6% (99/113), 85.8% (97/113) and 65.5% (74/113) in all patients, respectively. CA Ⅱ protein was strongly expressed in the cytoplasm of GIST cells (Figure 1B). Ki-67 protein was expressed in the nuclei of GIST cells (Figure 1D). In the control group, CA Ⅱ was negatively expressed in GIST cells (Figure 1C), and only partially expressed in neural astrocytoma, schwannoma, leiomyoma of the stomach, and malignant solitary fibrous tumors (Figure 1E-H). The histopathological type (spindle cell, epithelioid or mixed type) was noted and mitoses were counted using a × 40 objective for 50 high-power fields, as recommended.

Relationship between expression of CA Ⅱ, Ki-67 and CD117 and clinicopathological characteristics of GISTs

The survival analysis for all GIST patients showed that the 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0%. The recurrence rate was 10.6% with a recurrence time of 6-20 mo. The highest survival rate was found in those patients who received complete tumor resection and took imatinib (400 mg/d) postoperatively. However, in those patients who did not undergo complete tumor resection and were not treated with imatinib postoperatively, the survival rate was the lowest. However, in patients with positive CA Ⅱ or Ki-67 expression, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Considering molecular markers, the survival rates in the CA Ⅱ-negative group or CD117-negative group were significantly lower than in the positive groups (CA Ⅱ, log-rank $P = 0.000$; CD117, log-rank $P = 0.000$). However, it was higher in the Ki-67-positive group compared to the Ki-67-negative group (log-rank $P = 0.004$) (Figure 2).

According to the National Institutes of Health (NIH) risk grade[22] in GIST, there were five cases of extremely low risk, 15 of low risk, 16 of medium risk, and 77 of high risk. Comparing these parameters (tumor diameter, tumor site, mitotic rate, NIH risk, and depth of invasion), the differences were significant between the Ki-67-positive and -negative group, while for CD117 marker, there was no difference. For CA Ⅱ, significant differences were found between positive and negative groups only when they were compared by tumor diameter, mitotic rate and NIH risk. In the CA Ⅱ-positive group, high NIH risk accounted for 66.6% (66/99) of the cases (Figure 3). As for the other pathological characteristics, CD34, SMA and desmin protein positive rates were 79.4%, 46.8% and 5%, respectively.

When comparing tumor site, mitotic rate, NIH risk, and depth of invasion, the differences were significant between Ki-67-positive and -negative groups ($P < 0.05$), whereas for CD117, significant differences were found for age, tumor site and depth of invasion ($P < 0.05$). For CA Ⅱ, significant differences were not found between the positive and negative groups ($P > 0.05$) (Table 1).

Multivariate Cox model analysis suggested that CA Ⅱ, Ki-67 and CD117, along with tumor site, tumor diameter, mitotic rate, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors ($P < 0.05$). However, age, sex, mucosal erosion, biopsy and CD34 were not important prognosis predictive factors (Table 2).

DISCUSSION

It has been proved that tumor size, mitotic index, tumor location, and intraoperative tumor rupture are related to prognosis and recurrence of GIST[23-25]. For instance, although p53, CD147, MCT1, DDX39 and NKp30 are related to prognosis of GIST, they have never been mentioned as prognostic predictors due to their weak correlation.

Multivariate analysis showed that CA Ⅱ provided additional information on patient survival as compared to age, sex, NIH risk classification and mutational status. Based upon the comprehensive recognition that CA Ⅱ-positive tumor cells have oxidative activity, it is safe to suggest that CA Ⅱ plays an important role in occurrence and development of GIST. By contrast, various studies have included only the membrane-bound isoforms,

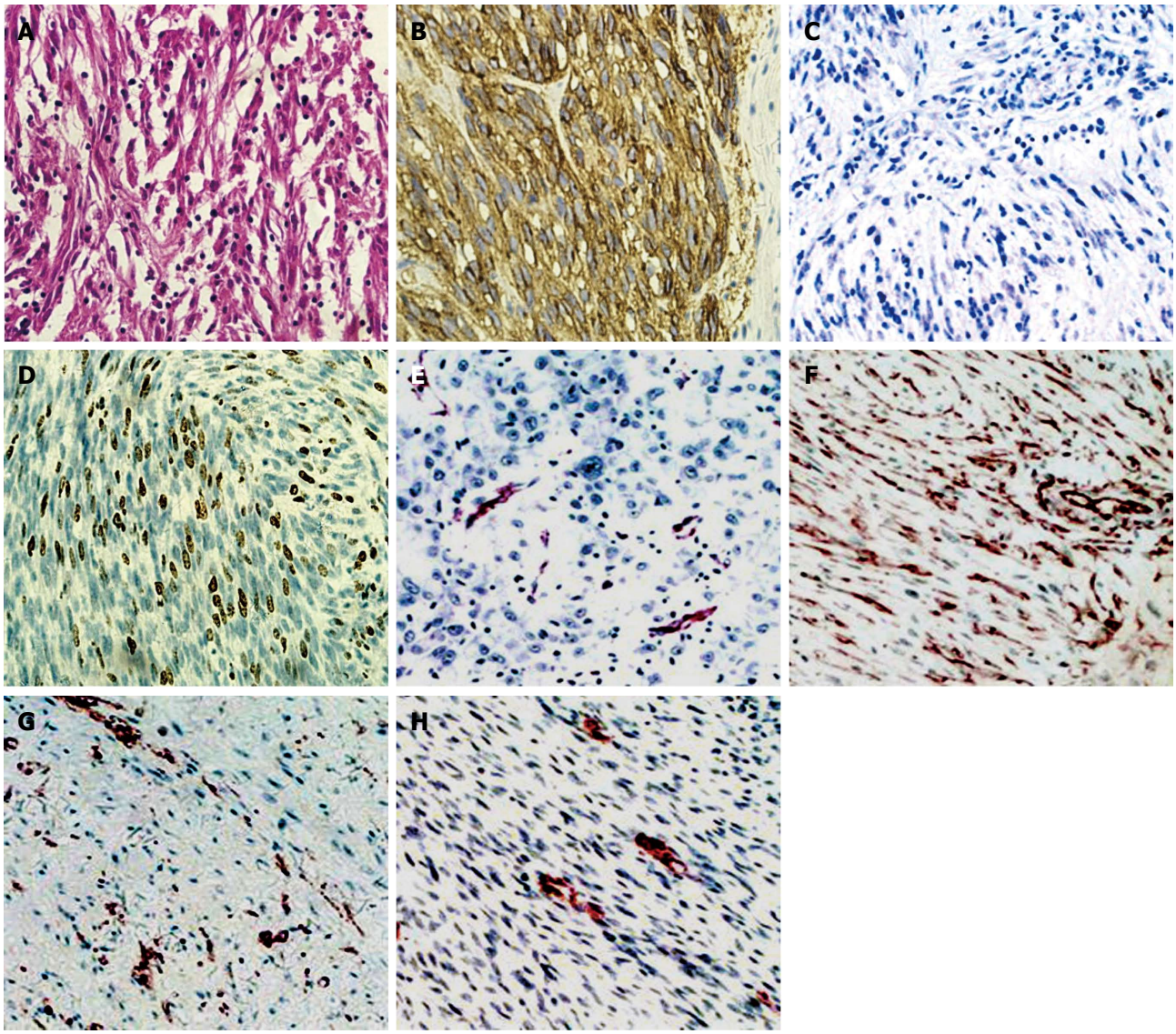

Figure 1 The scale is 5 μm using a × 400 objective as recommended. Carbonic anhydrase Ⅱ (CA Ⅱ) and Ki-67 proteins expressed in gastrointestinal stromal tumors (GISTs) and other malignant tumors. A: Hematoxylin and eosin in GISTs; B: CA Ⅱ positive protein in the GISTs; C: CA Ⅱ and Ki-67 negative protein in GISTs; D: Ki-67 positive protein in the GISTs; E: CA Ⅱ positive protein in neural astrocytoma; F: CA Ⅱ positive protein in schwannoma; G: CA Ⅱ positive protein in leiomyoma of the stomach; H: CA Ⅱ positive protein in malignant solitary fibrous tumor.

CA Ⅸ and Ⅻ, which are overexpressed in several types of cancers[26-29]. There has been only scattered evidence that CA Ⅱ is expressed to some extent in malignant cells such as leukemic blast cells, and brain, colorectal and pancreatic cancers[30-32]. A more recent study has indicated that CA Ⅱ expression is induced in neovascular endothelial cells of malignant melanoma and in esophageal, renal and lung cancers. It has been suggested that CA Ⅱ associated with the neovascular endothelia could serve as a potential target for cancer therapy. It has also been proposed that the presence of CA Ⅱ in the endothelium could contribute to generation of autoantibodies that could, in turn, be a desired outcome in immunotherapy of cancer. Combined with our present results, a new therapeutic approach targeting CA Ⅱ, as well as CA Ⅱ to predict prognosis of GIST, might be promising. However, more studies are necessary.

Ki-67 protein exists in actively proliferating cells (G1, S and G2 phase), which is a proliferation-related nuclear marker of tumor cell[33]. Some studies have shown that Ki-67 expression is closely related to aggressive biological behavior of tumor cells in GISTs[18] and represents a good prognostic predictor for GIST[34]. However, the significance of Ki-67 in predicting prognosis is still in dispute.

Wong *et al*[17] have found that Ki-67 was less reliable than mitotic count, even though it was useful in assessing the proliferation rate of tumor cells in GIST. We believe that the prognostic predictive value of Ki-67 in GIST might have been evaluated more objectively in a large survival study, with the various prognostic factors being taken into account. This was one of the aims of our present study. We found that the 1-, 3- and 5-year survival rates of patients with Ki-67-positive GIST were lower than in the Li-67-negative group. Our survival analysis further indicated that the Ki-67 expression was

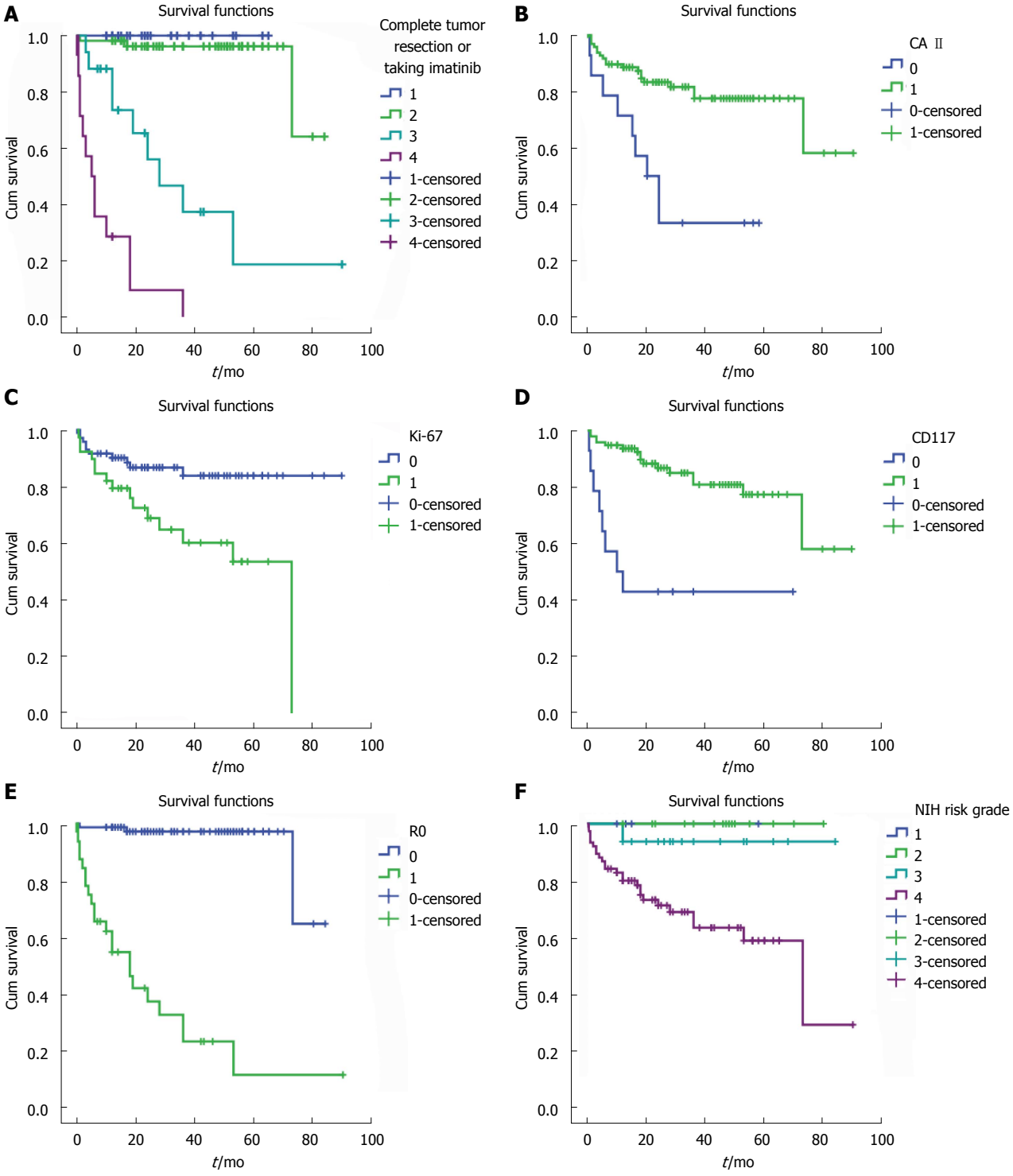

Figure 2 Analysis of survival rates (Kaplan-Meier) and comparison of survival rates in gastrointestinal stromal tumors groups (log-rank test). A: The highest survival rate was found in those patients that received complete tumor resection and postoperatively took imatinib (400 mg/d), while in those patients not receiving complete tumor resection and postoperative imatinib, the survival rate was lowest (log-rank $P = 0.000$). 1: Resected completely with imatinib administrated postoperatively, 2: Resected completely without imatinib administrated postoperatively, 3: Resected incompletely with imatinib administrated postoperatively, 4: Resected incompletely without imatinib administrated postoperatively; B: The survival rates in carbonic anhydrase (CA) Ⅱ-positive group were significantly higher than those in negative groups (CA Ⅱ, log-rank $P = 0.000$); C: The survival rates in Ki-67-positive group were higher than those in negative groups (Ki-67, log-rank $P = 0.004$); D: The survival rates in CD117-positive group were significantly higher than those in negative groups (CD117, log-rank $P = 0.000$); E: The higher survival rates were found in those patients that received complete tumor resection (R0) (R0, log-rank $P = 0.000$); F: The higher survival rates were found in those patients with National Institutes of Health (NIH) high risk (NIH risk, log-rank $P = 0.006$).

also an important prognostic predictor for GIST. The Wald indexes of Ki-67, diameter, mitotic rate and tumors site were all > 1 (7.282, 4.974, 11.081 and 15.581, respectively), which indicated that the Ki-67 was another useful molecular marker in predicting the prognosis of GISTs.

Table 1 Pathological parameters of gastrointestinal stromal tumors in CD117, carbonic anhydrase Ⅱ and Ki-67 proteins

Variable	Total	CD117		*P* value	CA Ⅱ		*P* value	Ki-67		*P* value
		+	-		+	-		+	-	
Sex										
Male	61	56	5	0.317	53	8	0.515	25	36	0.171
Female	52	41	11		46	6		14	38	
Age (yr)										
≤ 60	61	48	13	0.036	54	7	0.485	22	39	0.859
> 60	52	49	3		45	7		17	35	
Diameter										
≤ 5 cm	30	27	3	0.647	25	5	0.297	8	22	0.406
> 5 cm	83	70	13		74	9		31	52	
Site										
Stomach	45	40	5		41	4		13	32	
Small bowel	35	34	1	0.004	31	4	0.459	8	27	0.014
Others	33	23	10		27	6		18	15	
Mitotic rate										
≤ 5 MF/50 HPFs	52	45	7	0.941	46	6	0.515	11	41	0.009
> 5 MF/50 HPFs	61	52	9		53	8		28	33	
NIH risk										
Very low	5	4	1		4	1		2	3	
Low	15	13	2	0.980	13	2	0.424	2	13	0.031
Medium	16	14	2		16	0		2	14	
High	77	66	11		66	11		33	44	
Depth of invasion										
Mucosa	24	21	3		21	3		6	18	
Muscular	41	33	8		38	3		10	31	
Serous	30	30	0	0.033	27	3	0.168	13	17	0.013
Adjacent tissue	18	13	5		13	5		10	8	

This table shows that Being compared by tumor site, mitotic rate, National Institutes of Health (NIH) risk, and depth of invasion of tumor cells, the differences were significant between the Ki-67-positive and -negative groups, while for CD117, significant differences were found for age, tumor site and depth of invasion of tumor cells. For carbonic anhydrase (CA) Ⅱ, no significant differences were found between positive and negative groups. MF: Mitotic figures; HPFs: High-power fields.

Table 2 Multivariate survival analysis (Cox proportional hazards model) in gastrointestinal stromal tumors

Variable	*B*	Wald	*df*	*P* value	HR	95%CI for HR	
						Lower	Upper
Age	0.023	1.696	1	0.193	1.024	0.988	1.060
Sex	-0.239	0.342	1	0.559	0.788	0.354	1.754
Mucosal erosion or not	1.485	2.115	1	0.146	4.416	0.597	32.684
Biopsy or not	-0.404	0.901	1	0.340	0.668	0.291	1.531
CD34	-0.071	0.086	1	0.769	0.932	0.582	1.493
CA Ⅱ	-0.319	4.113	1	0.043	0.727	0.543	0.989
CD117	-0.609	4.114	1	0.042	0.544	0.303	0.978
Ki-67	1.103	7.282	1	0.007	3.014	1.352	6.717
Diameter	1.645	4.974	1	0.026	5.182	1.216	22.085
Mitotic rate	0.972	11.081	1	0.001	2.644	1.491	4.686
Site	1.334	15.581	1	0.000	3.796	1.955	7.371
Depth of invasion	0.559	7.445	1	0.006	1.748	1.170	2.612
Complete resection or not	2.807	24.674	1	0.000	16.555	5.470	50.104
Intraoperative rupture	1.937	17.997	1	0.000	0.144	0.059	0.353
Adjuvant therapy	1.757	35.579	1	0.000	5.796	3.254	10.325

This table shows that carbonic anhydrase (CA) Ⅱ, CD117 and Ki-67 expression, tumor diameter, mitotic rate, tumor site, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors. HR: Hazard ratios.

As for molecular markers, the negative expression of CD117 is believed to be associated with early postoperative recurrence of GIST[35]. This was confirmed once again by our study. At the same time, our study indicated that positive expression of Ki-67 or negative expression of CA II and CD117 was a cue for poor prognosis in GIST. However, there was a limitation to our study, namely, its small sample size. Our results could promote the clinical application of these two markers and provide clues to a novel therapeutic target for GIST in the future.

In conclusion, our study showed CA Ⅱ expression in GIST. The prognosis of GIST with high CA Ⅱ expres-

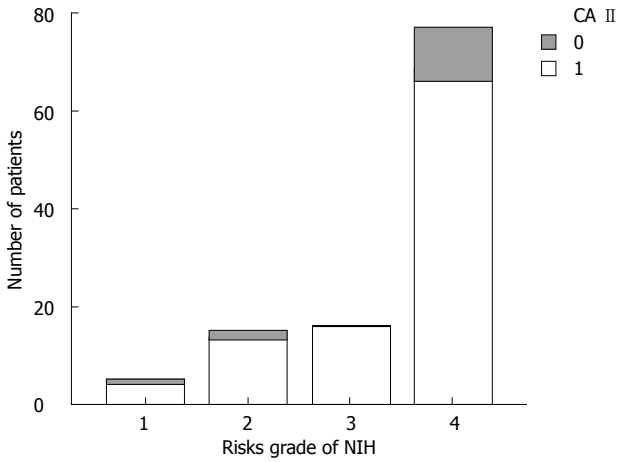

Figure 3 Carbonic anhydrase Ⅱ positive cases in gastrointestinal stromal tumors according to National Institutes of Health risk grade. There were five cases with extremely low risk, 15 with low risk, 16 with medium risk, and 77 with high risk. Carbonic anhydrase (CA) Ⅱ expressed in 113 gastrointestinal stromal tumors: "0" represents the negative cases, and "1" the positive cases. NIH: National Institutes of Health.

sion was better than that of GIST with low or no expression, suggesting that CA Ⅱ is both a diagnostic and prognostic biomarker. Further validation studies with other CA antibodies should be undertaken to characterize CA Ⅱ expression in a larger cohort of patients with GIST and other mesenchymal tumors of the gastrointestinal tract.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract with a wide spectrum of clinical behavior. Thus, it is of great importance to define prognostic factors that could indicate survival rates. Carbonic anhydrase (CA) Ⅱ is expressed in many malignant tumors, and higher expression is associated with a better disease-specific survival rate. Ki-67 is related to cell proliferation in various tumors and has a markedly positive correlation with prognosis.

Research frontiers

According to National Institutes of Health risk classification, some prognostic factors are well documented as prognostic factors of further tumor behavior, such as mitotic count and tumor size, along with tumor primary localization. However, the value of immunohistochemistry index in GISTs has not been clearly indicated. Furthermore, there have been a limited number of studies investigating the relationship between the prognosis of GISTs and CA Ⅱ and Ki-67.

Innovations and breakthroughs

This is believed to be the first study showing so clearly the significance of CA Ⅱ and Ki-67 as prognostic factors. The 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0% in all patients. However, in patients with positive CA Ⅱ or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. This study indicated that CA Ⅱ has strong expression in GISTs and the prognosis of GISTs with high CA Ⅱ expression was better than that of GISTs with low or no expression, suggesting that CA Ⅱ is both a diagnostic and a prognostic biomarker for GIST.

Applications

CA Ⅱ and Ki-67 were useful for predicting the aggressive biological behavior of GISTs. CA Ⅱ associated with neovascular endothelia could serve as a potential target for cancer therapy.

Terminology

CAs are a group of zinc-containing metalloenzymes that catalyze the reversible hydration of CO_2 and participate in various physiological processes, including respiration, gluconeogenesis, bone resorption, renal acidification, and formation of cerebrospinal fluid and gastric acid. Ki-67 is a protein that is encoded by the *MKI-67* gene in humans. Ki-67 antigen is associated, and probably necessary, for cellular proliferation, and is associated with rRNA transcription.

Peer review

This study revealed that CA Ⅱ is highly expressed in GIST cell lines and 87.6% of GISTs selectively. Until now, there have been few reports of CA Ⅱ expression in GISTs. The result of this study showed that high CA Ⅱ expression was associated with a better disease-specific survival rate than low or no expression, therefore, it might be a useful biomarker in diagnosis and prognosis of GISTs. This is a retrospective study and the results should be helpful for clinical practice and a potential therapeutic target.

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ORIGINAL ARTICLE

Emodin regulating excision repair cross-complementation group 1 through fibroblast growth factor receptor 2 signaling

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Abstract

AIM: To investigate the molecular mechanisms underlying the reversal effect of emodin on platinum resistance in hepatocellular carcinoma.

METHODS: After the addition of 10 μmol/L emodin to HepG2/oxaliplatin (OXA) cells, the inhibition rate (IR), 50% inhibitory concentration (IC_{50}) and reversal index (IC_{50} in experimental group/IC_{50} in control group) were calculated. For HepG2, HepG2/OXA, HepG2/OXA/T, each cell line was divided into a control group, OXA group, OXA + fibroblast growth factor 7 (FGF7) group and OXA + emodin group, and the final concentrations of FGF7, emodin and OXA in each group were 5 ng/mL, 10 μg/mL and 10 μmol/L, respectively. Single-cell gel electrophoresis was conducted to detect DNA damage, and the fibroblast growth factor receptor 2 (FGFR2), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and excision repair cross-complementing gene 1 (ERCC1) protein expression levels in each group were examined by Western blotting.

RESULTS: Compared with the IC_{50} of 120.78 μmol/L in HepG2/OXA cells, the IC_{50} decreased to 39.65 μmol/L after treatment with 10 μmol/L emodin; thus, the reversal index was 3.05. Compared with the control group, the tail length and Olive tail length in the OXA group, OXA + FGF7 group and OXA + emodin group were significantly increased, and the differences were statistically significant ($P < 0.01$). The tail length and Olive tail length were lower in the OXA + FGF7 group than in the OXA group, and this difference was also statistically significant. Compared with the OXA + FGF7 group, the tail extent, the Olive tail moment and the percentage of tail DNA were significantly increased in the OXA + emodin group, and these differences were statistically significant ($P < 0.01$). In comparison with its parental cell line HepG2, the HepG2/OXA cells demonstrated significantly increased FGFR2, p-ERK1/2 and ERCC1 expression levels, whereas the expression of all three molecules was significantly inhibited in HepG2/OXA/T cells, in which FGFR2 was silenced by FGFR2 shRNA. In the examined HepG2 cells, the FGFR2, p-ERK1/2 and ERCC1 expression levels demonstrated increasing trends in the OXA group and OXA + FGF7 group. Compared with the OXA group and OXA + FGF7 group, the FGFR2, p-ERK1/2, and ERCC1 expression levels were significantly lower in the OXA + emodin group, and these differences were statistically significant. In the HepG2/OXA/T cell line that was transfected with FGFR2 shRNA, the FGFR2, p-ERK1/2 and ERCC1 expression levels were significantly inhibited, but there were no significant differences in these

expression levels among the OXA, OXA + FGF7 and OXA + emodin groups.

CONCLUSION: Emodin markedly reversed OXA resistance by enhancing OXA DNA damage in HepG2/OXA cells, and the molecular mechanism was related to the inhibitory effect on ERCC1 expression being mediated by the FGFR2/ERK1/2 signaling pathway.

Key words: Hepatocellular carcinoma; Emodin; Fibroblast growth factor receptor 2; Excision repair cross-complementation group 1; Platinum resistance; Extracellular signal-regulated kinase

Core tip: In this study, our results indicated that emodin could significantly enhance the DNA damage caused by oxaliplatin (OXA) and induce OXA resistance reversal in HepG2/OXA cells. The molecular mechanism for this phenomenon is mediated by the inhibition of excision repair cross-complementing gene 1 expression by the fibroblast growth factor receptor 2/phosphorylated extracellular signal-regulated kinase 1/2 signaling pathway. The results for the reversal of platinum resistance by emodin and the emodin-based enhancement of the efficacy of platinum-based chemotherapy in hepatocellular carcinoma may provide an experimental basis for the further development and application of emodin in the reversal of platinum drug resistance in other types of malignant tumors.

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INTRODUCTION

For up to 85%-95% of the cases of liver cirrhosis in patients suffering from hepatocellular carcinoma (HCC), the therapeutic effects of chemotherapy have not been proven to provide survival benefits[1]. For the patient and the oncologist, sorafenib, which is a unique targeting drug that generates proven survival benefits, does not produce many unexpectedly positive outcomes because of its limited cost-effectiveness[2]. In 2010, at the annual meeting of the American Society of Clinical Oncology, the results of an international multi-center randomized phase Ⅲ clinical study provided the first evidence demonstrating that platinum-based chemotherapy produces survival benefits for patients with advanced HCC. This discovery has triggered widespread interest into the use of oxaliplatin-based chemotherapy for HCC[3]. Thus, the identification and characterization of a drug that strengthens platinum-based chemotherapy effects and protects normal liver cells could provide clinical benefits for the treatment of HCC in China.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a member of the family of anthraquinone alkaloids, which are the main active ingredients of rhubarb, *Polygonum cuspidatum*, *Polygonum multiflorum*, *Amomum*, lilies and other plants that are widely used in traditional Chinese medicine[4]. Modern studies have demonstrated that emodin produces anti-tumor biological effects on a variety of malignancies, including HCC[5]. In HCC, the primary anti-cancer mechanisms of emodin involve the induction of apoptosis and the inhibition of cell growth. Emodin can cause G2/M cell cycle arrest by regulating an assortment of cell cycle related genes, such as *cyclin A*, *cyclin B*, *Chk2*, *CDK2* and *p27*, in diverse human hepatoma cell lines, including Huh7, Hep3B and HepG2[6]. Moreover, emodin can enhance the cytotoxicity of chemotherapy drugs, such as platinum-based compounds [cisplatin, carboplatin, and oxaliplatin (OXA)] in various types of malignant tumors, including liver cancer, gallbladder cancer[7,8], non-small cell lung cancer[9,10], and prostate cancer[11]. However, the mechanism underlying the synergistic effects of combinations of emodin and platinum-based drugs requires further elucidation.

The DNA damage that is caused by cisplatin, OXA and other platinum chemotherapy drugs is the root cause of the cytotoxicity of these compounds[12]. Nucleotide excision repair (NER) is the main pathway for repairing this damage, and excision repair cross-complementing gene 1 (*ERCC1*), which is the limiting enzyme in the NER pathway, plays an important role in this process[13,14]. Compared with normal liver tissue, fibrous tissue in the liver displays significantly increased levels of ERCC1 expression[15]. ERCC1 protein concentrations were significantly greater in liver cancers that were accompanied by hepatic fibrosis tissue than in liver cancers without hepatic fibrosis. In addition, high expression levels of ERCC1 are closely correlated with cisplatin resistance; thus, ERCC1 could be used as a predictor of sensitivity to platinum-based chemotherapy in cases of HCC[16]. The relationship between synergistic effects and the regulation of ERCC1 expression is worthy of further study in HCC treatments that combine emodin with platinum drugs.

Fibroblast growth factor receptor 2 (FGFR2), which is a member of a transmembrane tyrosine kinase receptor family (FGFRs), is an expression product of the bek oncogene that plays an important role in the differentiation of HCC, the clinical staging of tumors, the incidence of tumor thrombosis and the determination of alpha-fetoprotein levels[17]. As a molecular marker, FGFR2 can effectively predict the overall survival and progression-free survival of patients with HCC. Interestingly, *ERCC1* is a downstream target gene of FGFR2[18], whereas emodin is a tyrosine kinase inhibitor[10,19]. Previously published research has indicated that emodin down-regulates ERCC1 expression in non-small cell lung cancer, and its effects may be relevant to the ERK1/2 signaling pathway[20]; however, the exact mechanism

through which emodin produces these effects has not been well established. The ERK signaling pathway is one of the downstream components of the FGFR2 pathway[21,22]. Thus, we hypothesize that emodin reverses tumor drug resistance by increasing the DNA damage that is induced by platinum chemotherapy drugs, and we speculate that the molecular mechanism of this effect is related to the ERK1/2 pathway, which is mediated by FGFR2 signaling in hepatoma cells.

The primary aim of this study was to determine the molecular mechanisms underlying the reversal effect of emodin on platinum resistance in HCC.

MATERIALS AND METHODS

Oxaliplatin and emodin were purchased from Sigma (St. Louis, MO, United States). Dulbecco's modified Eagle's medium/high glucose (DMEM/H) and fetal bovine serum were produced by Invitrogen (Carlsbad, CA, United States), trypsin and dimethyl sulfoxide was purchased from Guge Biology CO (Wuhan, China). FGF7, puromycin dihydrochloride (sc-108071), ERCC1 mouse anti-monoclonal antibody and p-ERK1/2 rabbit anti-monoclonal antibody (sc-16982-R) were purchased from Santa Cruz Biotechnology (United States). FGFR2 mouse anti-monoclonal antibody was produced (MAB684) by R-D Systems, Inc. (United States). Dylight™ 800-labeled anti-mouse immunoglobulin G (IgG) antibody and Dylight™ 800-labeled anti-rabbit IgG antibody were bought from Gaithersburg Biotechnology (MD, United States).

Cell lines and culture conditions

The human hepatoma cell line HepG2 was used in the present study. HepG2 cells were obtained from the China Center for Type Culture Collection. The HepG2 cells were cultured in DMEM/H supplemented with 10% (v/v) fetal bovine serum, 200 IU/mL penicillin (ICN Biomedical, Costa Mesa, CA, United States), 100 mg/mL streptomycin (ICN Biomedical) and 0.5 mmol/L sodium pyruvate (Cambrex, Walkersville, MD, United States). The cells were cultured at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. An OXA-resistant subline was established by discontinuously exposing parental HepG2 cells to high OXA concentration (25 μmol/L) medium over the course of one year until the resulting cells could grow exponentially in medium containing 1 μmol/L OXA. The HepG2/OXA cells were digested and subcultured three times prior to their use for this experiment.

Reversal effect of emodin on HepG2/OXA

Cells from the resistant cell line HepG2/OXA in logarithmic growth phase were grown in 96-well plates. In particular, 100 μL of cell suspension was inoculated into 8 mL of medium. Medium containing different concentrations of the chemotherapy drug OXA (3.125, 6.25, 12.5, 25, 50 or 100 μmol/L) was added after 12 h of culture. The experimental group (EG) also received a final concentration of 10 μmol/L emodin in medium. In addition, a control group (CG) was established. The medium was aspirated after 24 h of culture; subsequently, 110 μL of a mixture of DMEM/H and cell counting kit-8 (CCK-8) (at a ratio of 10 μL CCK-8:100 μL DMEM/H) was added, after which the samples were incubated for 2 h. A cell-free blank group (BG) was established. The optical density (OD) of each well was determined at 450 nm. The formula for calculating the inhibition rate (IR) at different concentrations was $1 - (OD_{EG} - OD_{BG})/(OD_{CG} - OD_{BG})$. Based on the IR at different concentrations of the anti-cancer drug, the concentration at which the inhibition rate was 50% (IC_{50}) was calculated using the SPSS 13.0 software (SPSS Inc., Chicago, IL, United States). The reversal index was calculated using the formula (IC_{50EG}/IC_{50CG}). Five wells were established at different experimental concentrations, and each experiment was repeated three times.

Short hairpin RNA for bek gene cell transfection in HepG2/OXA

Bek shRNA cell transfection was conducted in accordance with the protocol for the bek shRNA plasmid (h): sc-29218-S (Santa Cruz Biotechnology, Inc.). HepG2/OXA cells were cultured to 60%-80% adherence in 6-well cell culture plates by adding antibiotic-free fetal bovine serum growth medium. The shRNA plasmid DNA solution (Solution A) was added directly to the dilute shRNA plasmid transfection reagent (Solution B) using a pipette. The solution was mixed gently by pipetting up and down and was incubated for 30 min at room temperature. Subsequently, the cells were washed twice with 2 mL of shRNA transfection medium, after which the medium was aspirated. Immediately afterward, 200 μL shRNA plasmid DNA/shRNA plasmid transfection reagent was added. The cells were incubated for 6 h at 37 ℃ in a CO_2 incubator. Following this incubation, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added to each well, and the cells were incubated for an additional 24 h under normal conditions. Forty-eight hours after the transfection, the medium was aspirated and replaced with fresh medium containing 5 μg/mL puromycin. Every 2 d afterward, the medium was aspirated and replaced with freshly prepared selective medium. Finally, an aliquot of the cells was washed once with phosphate buffer saline (PBS). The cell sample was lysed in 300 μL 1 × electrophoresis sample buffer by gently rocking the 6-well plate and was subjected to sodium dodecyl sulfate (SDS) gel electrophoresis to confirm the silencing of the *FGFR2* gene. Thus, a Bek-silenced HepG2/OXA cell line (HepG2/OXA/T) was established.

Experimental groups and sample preparation

HepG2, HepG2/OXA and HepG2/OXA/T cells were seeded in culture flasks. Each cell type was divided into four groups: a CG, an OXA group, an OXA + FGF7 group and an OXA + emodin group. A final concentration of 10 μmol/L OXA was added to the OXA group,

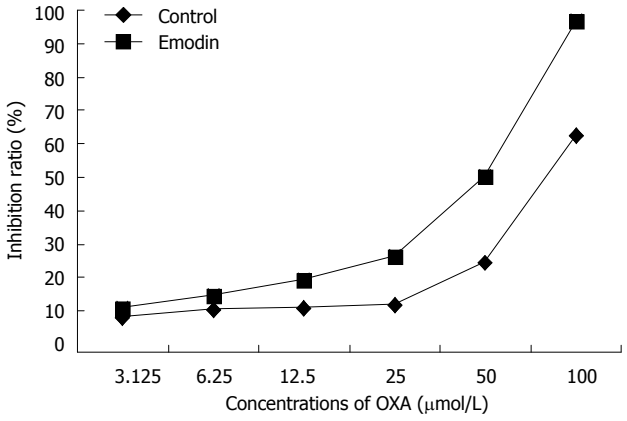

Figure 1 Inhibition ratio of different concentrations (μmol/L) of oxaliplatin in HepG2/oxaliplatin cells. The value of inhibition ratio increased steadily in the control group and the emodin group, which accompanied the elevated concentration of oxaliplatin (OXA) in the HepG2/OXA cells. However, the value of inhibition ratio in the emodin group treated with 10 μmol/L emodin was significantly higher in comparison with the control group.

the OXA + FGF7 group and the OXA + emodin group. and the final concentrations of FGF7, emodin and OXA in each group were 5 ng/mL, 10 μg/mL and 10 μmol/L, respectively. After being incubated for 24 h and digested with trypsin, the cells were divided into two portions, one for total protein preparation for Western blotting, and one for single-cell gel electrophoresis to assay the DNA damage.

Single-cell gel electrophoresis to detect DNA damage

After counting the cells and adjusting them to a concentration of 2000 cells/μL, the cells in each group were gently suspended into single-cell suspension. Subsequently, 110 μL of 0.5% normal melting point agarose (NMA) at 45 ℃ was poured onto Dakin slides, avoiding the production of air bubbles. The agarose solidified at room temperature. Subsequently, 5 μL PBS containing 10000 cells in each group was mixed well with 75 μL of 0.5% low melting point agarose (LMA). The upper cover-slip was carefully removed, the mixture was quickly added to the 0.5% NMA, the cells were spread evenly, and the slide was placed in a 4 ℃ refrigerator for 5 min until the agarose solidified. The cover-slip was removed, and 75 μL of 0.5% LMA was added, after which the slide was placed in the refrigerator at 4 ℃ again to solidify the agarose. The slide was slowly immersed in freshly prepared 4 ℃ pre-cooling cell lysate and then set in a 4 ℃ refrigerator for at least 1 h. The slide was then placed in a horizontal gel electrophoresis tank and incubated in the dark for 45 min, after which electrophoresis was performed for 30 min at 25 V at room temperature, and the height of the electrophoresis buffer liquid was adjusted to maintain a continuous 300 mA current. After the electrophoresis, each slide was dipped two times in a buffer in a darkroom for 5 min each. Following this treatment, 0.5 μL ethidium bromide dye was added to stain the DNA, the cover-slip was stamped, and the slide was observed and analyzed under a fluorescence microscope with a camera.

Protein expression of FGFR2, ERCC1, p-ERK1/2 and β-actin by Western blotting

Total protein was collected from the different groups of the cultured HepG2, HepG2/OXA and HepG2/OXA/T cells. The protein concentration was measured by a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Before electrophoresis, the protein was denatured in lithium dodecyl sulfate (LDS) sample buffer (106 mmol/L Tris-HCl, 141 mmol/L Tris base pH 8.5, 0.51 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 2% LDS, 0.22 mmol/L Serva blue G250, 0.175 mmol/L phenol red, and 0.1 mmol/L 2-mercaptoethanol) for 10 min at 95 ℃. The total protein (20 μg per lane) was electrophoresed on a 8% SDS polyacrylamide gel electrophoresis gel and transferred onto a 0.45 μm nitrocellulose filter membrane (Roche, Indianapolis, IN, United States). The membranes were blocked with 5% (w/v) nonfat dry milk in PBS containing 0.05% Tween-20 (PBST) for 2 h at room temperature and incubated overnight at 4 ℃ with antibodies against FGFR2 (1:250), p-ERK1/2 (1:250) or ERCC1 (1:100) (Santa Cruz, CA, United States). Next, the membranes were incubated with a Dylight™ 800-labeled antibody for 1 h after being washed 4 times for 5 min in PBST. Finally, the immunoblot signals were scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Nebraska, United States).

Statistical analysis

All of the digital results are displayed as the means ± SD. The quantitative ratios of different groups were compared using Student's t-test with the SPSS 13.0 software. Probability values of $P < 0.05$ were regarded as statistically significant. All of the statistical tests were two-sided.

RESULTS

The reversal effect of emodin on platinum resistance in the HepG2/OXA cell line

Compared with the CG, in which HepG2/OXA was treated with OXA alone, the inhibition ratio was significantly increased in the emodin group, in which HepG2/OXA cells were treated with a combination of OXA and emodin (Figure 1). Based on the inhibition ratios of different concentrations (μmol/L) of OXA, the IC_{50} of the OXA-resistant HepG2/OXA cells was 120.78 μmol/L; however, in the OXA-resistant HepG2/OXA cells that were treated with 10 μmol/L emodin, the IC_{50} was reduced to 39.65 μmol/L. This result indicated that the reversal index of 10 μmol/L emodin in HepG2/OXA cells was 3.05 (Table 1).

DNA damage detected by single-cell gel electrophoresis

Compared with the CG of HepG2 cells, the tail extent (TE) and the Olive tail moment (OTM) were considerably increased in the OXA group, OXA + FGF7 group, and OXA + emodin group; these differences were statistically significant ($P < 0.01$). Compared with the OXA group,

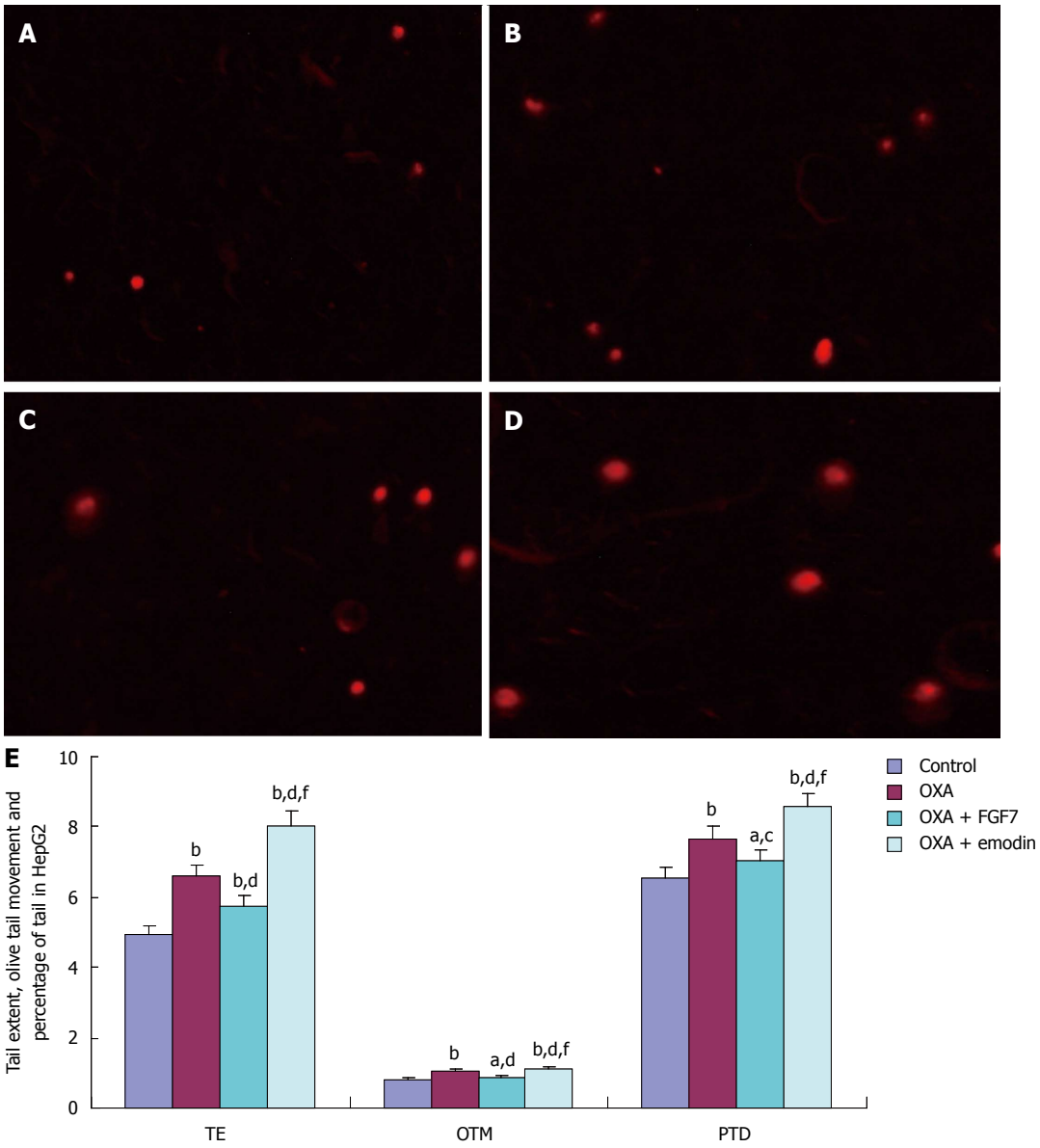

Figure 2 DNA damage detected by single cell gel electrophoresis in HepG2. A-D: Ethidium bromide stain (magnification × 200). Control group (A); Oxaliplatin (OXA) group (B); OXA + fibroblast growth factor 7 (FGF7) group (C); OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF7 group, respectively, and these differences were statistically significant. TE, OTM and PTD were significantly lower in the OXA + FGF7 group compared with OXA + emodin group and OXA group; however, these values were higher than those of the control group ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{e}P < 0.01$ *vs* OXA group; $^{f}P < 0.01$ *vs* OXA + FGF7 group).

TE and OTM in the OXA + FGF7 group were considerably decreased, and these differences were statistically significant ($P < 0.01$). TE, OTM and the percentage of tail DNA (PTD) were significantly greater ($P < 0.01$) in the OXA + emodin group than in the OXA + FGF7 group. PTD in the CG, OXA group, and OXA + FGF7 group did not significantly differ ($P > 0.05$) (Figure 2).

In HepG2/OXA cells, compared with the CG, TE, PTD and OTM in the OXA group, OXA + FGF7 group and OXA + emodin group were significantly increased; the differences were statistically significant ($P < 0.01$). Compared with the OXA group, OTM and PTD in the OXA + FGF7 group were reduced with statistically significant difference ($P < 0.01$). In the OXA + emodin group, TE, OTM and PTD were significantly increased; this difference was statistically significant ($P < 0.01$). Compared with the OXA + FGF7 group, TE, OTM and PTD in the OXA + emodin group were significantly increased ($P < 0.01$). These results are presented in Figure 3.

In HepG2/OXA/T cells, compared with the CG, TE, OTM and PTD in the OXA group, OXA + FGF7 group, and OXA + emodin group were significantly higher; the differences were statistically significant. Compared with the OXA group, OTM and PTD in the OXA + FGF7 group were reduced, and there was a significant difference; however, TE, OTM and PTD were significantly increased in the OXA + emodin group, with a statistically significant difference ($P < 0.01$). In comparison with the OXA + FGF7 group, TE, OTM and PTD were significantly increased in the OXA + emodin group, and the

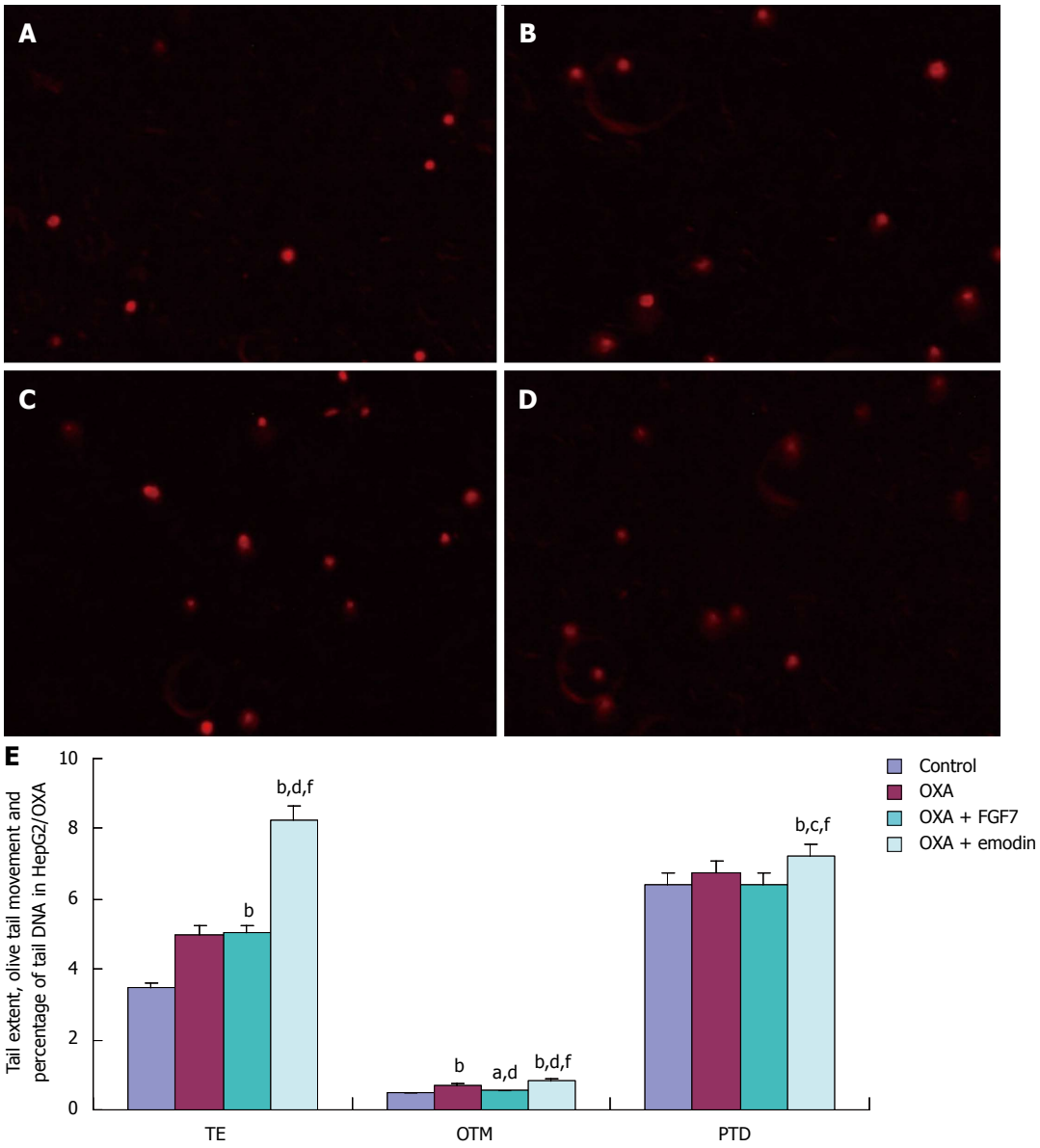

Figure 3 DNA damage detected by single cell gel electrophoresis in HepG2/oxaliplatin. A-D: Ethidium bromide stain (magnification × 200). Control group (A); Oxaliplatin (OXA) group (B); OXA + fibroblast growth factor 7 (FGF7) group (C); OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF group, respectively, and these differences were statistically significant. As for PTD there was no significant difference between the OXA group and the OXA + FGF7 group ($^aP < 0.05$, $^bP < 0.01$ *vs* control group; $^cP < 0.05$, $^dP < 0.01$ *vs* OXA group; $^fP < 0.01$ *vs* OXA + FGF7 group).

Table 1 Inhibition ratio and 50% inhibitory concentration, and reversal index of 10 μmol/L emodin in HepG2/oxaliplatin (mean ± SD) (n = 5)

OXA concentration (μmol/L)	3.125	6.25	12.5	25	50	100	IC_{50} (μmol/L)	Reversal index
IR in control (%)	9.76 ± 1.18	11.94 ± 1.30	13.95 ± 1.11	14.58 ± 1.02	28.06 ± 2.01	63.95 ± 4.71	120.78 ± 9.68	3.05
IR in emodin (%)	12.35 ± 1.3[a]	16.76 ± 1.3[a]	21.69 ± 1.6[a]	29.87 ± 1.55[b]	48.14 ± 2.09[b]	80.34 ± 3.00[b]	39.65 ± 5.43	

$^aP < 0.05$, $^bP < 0.01$ *vs* control group. IR: Inhibition ratio; OXA: Oxaliplatin; IC_{50}: 50% inhibitory concentration.

difference was statistically significant ($P < 0.01$), as shown in Figure 4.

FGFR2, pERK1/2, and ERCC1 protein expression

In comparison with the expression levels in the parental HepG2 cell line, the FGFR2, pERK1/2 and ERCC1 expression levels in the resistant cell line HepG2/OXA were significantly increased, whereas the pERK1/2 and ERCC1 expression levels in the shRNA-transfected cell line HepG2/OXA/T were significantly inhibited, as FGFR2 expression was silenced. Compared with the CG in HepG2 cells, FGFR2 expression was increased

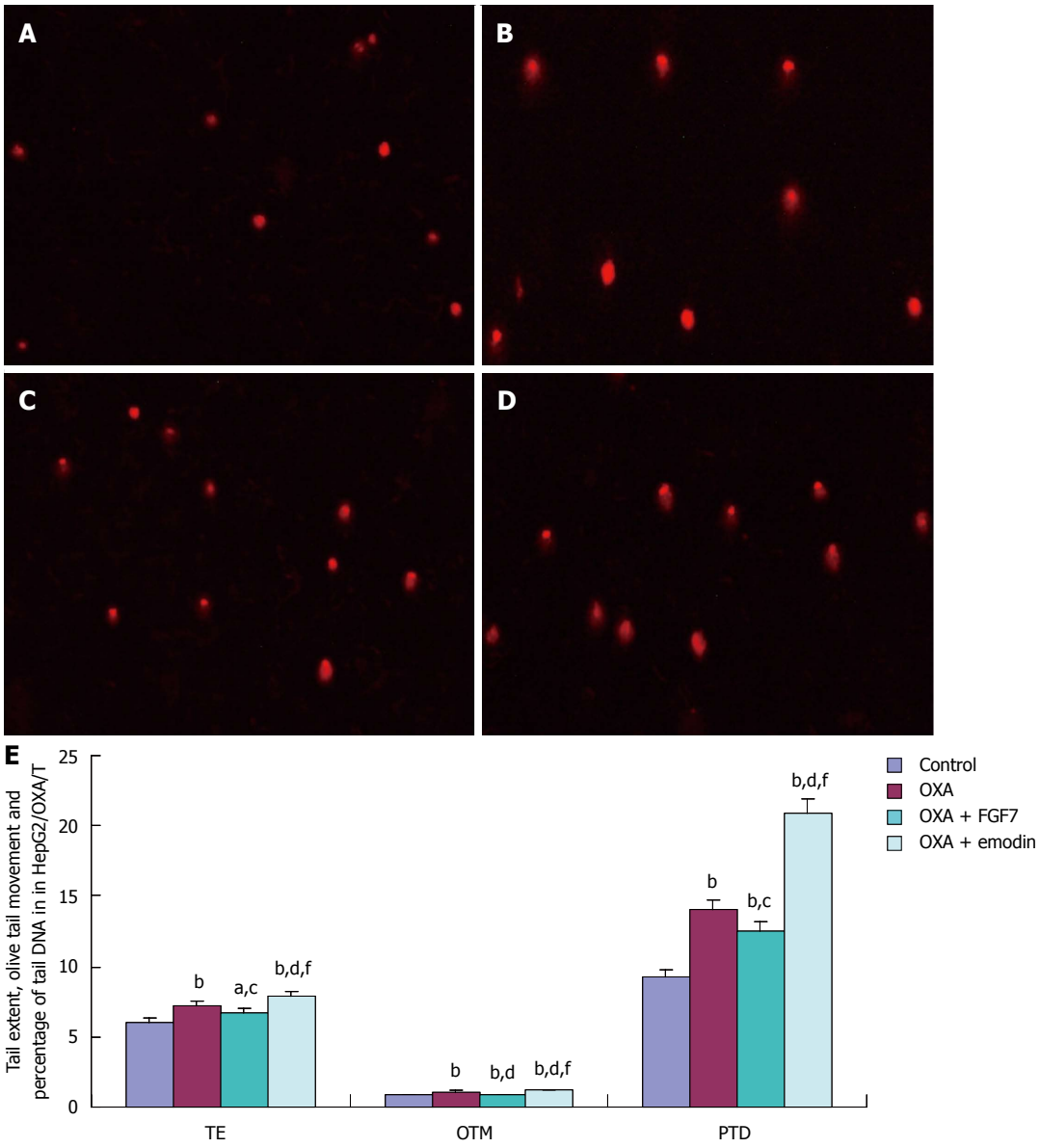

Figure 4 DNA damage detected by single cell gel electrophoresis in HepG2/oxaliplatin/T. A-D: Ethidium bromide stain (magnification × 200). Control group (A), Oxaliplatin (OXA) group (B), OXA + fibroblast growth factor 7 (FGF7) group (C), OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF7 group, respectively, and these differences were statistically significant. TE, OTM and PTD were significantly decreased in the OXA + FGF7 group in comparison to those in the OXA group and the OXA + FGF7 group, but were greater than those in the control group ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* OXA group; $^{f}P < 0.01$ *vs* OXA + FGF7 group).

with statistical significance in the OXA + FGF7 treatment group; moreover, there was a significant difference between the OXA + emodin group and the OXA + FGF7 group. The same trend was observed in the HepG2 and HepG2/OXA cells with respect to the expression of pERK1/2 and ERCC1 among the different treatment groups. Compared with the pERK1/2 and ERCC1 expression in CG, the levels in the OXA group were significantly up-regulated. Compared with the CG, pERK1/2 and ERCC1 expression were increased in the OXA + FGF7 group and reduced in the OXA + emodin group. However, the expression of FGFR2, pERK1/2 and ERCC1 exhibited no significant differences among the treatment groups in the shRNA-transfected cell line HepG2/OXA/T (Figure 5).

DISCUSSION

Rhubarb and polygonum cuspidatum have been widely used in various heat syndromes to clear heat and detoxify in the body in accordance with the theories of traditional Chinese medicine. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), the primary active ingredient in these traditional medicines, was identified by modern pharmacological studies as having a wide range of pharmacological effects, such as protecting the function of the liver and the kidney[23], producing anti-inflammatory effects[24] and regulating lipid metabolism[25,26]. In recent years, its anti-cancer function has been revealed in a variety of malignancies, including HCC[27]. In addition, combined with chemotherapy drugs such as platinum[7-11,16], emodin

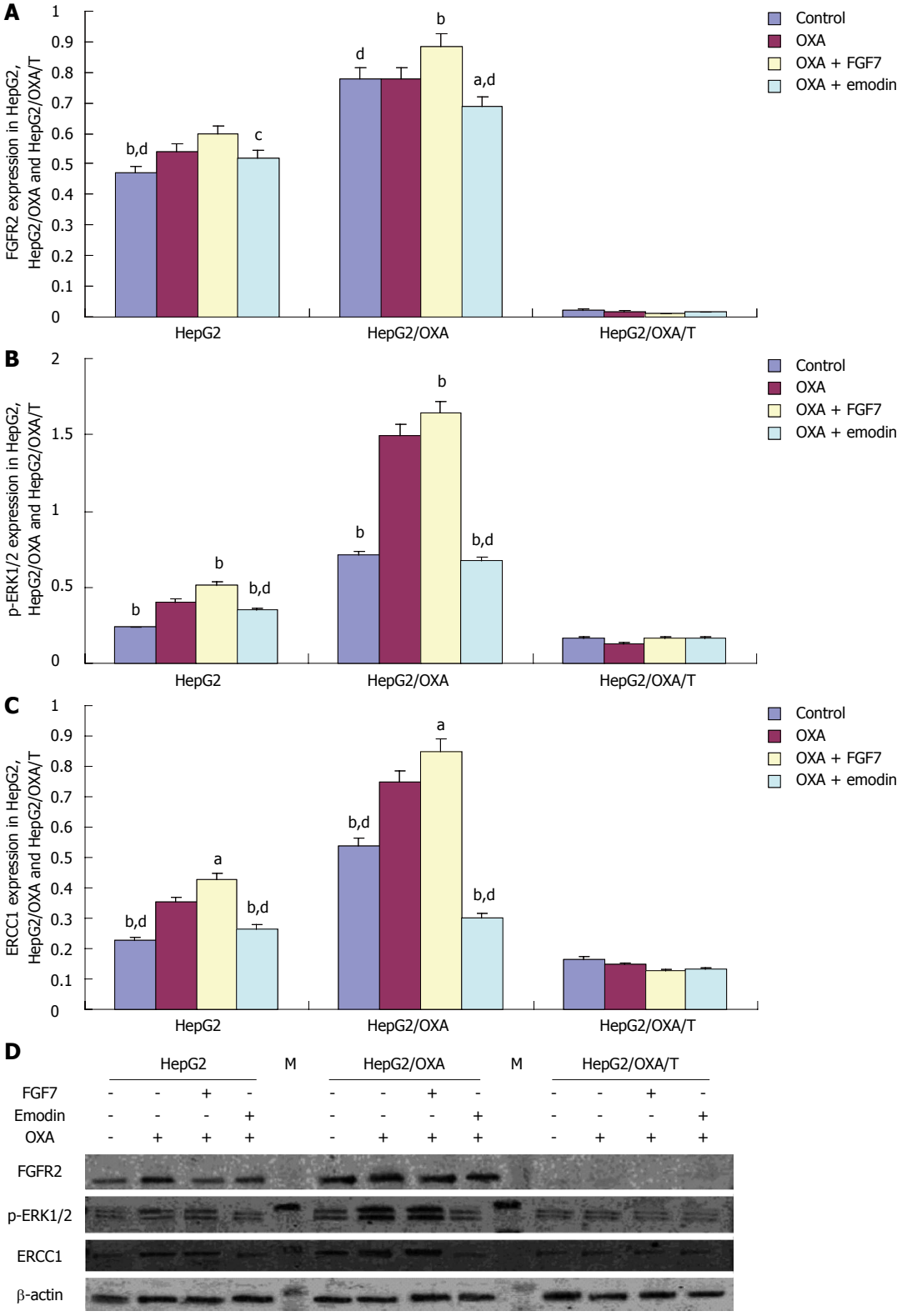

Figure 5 Comparison of fibroblast growth factor receptor 2, phosphorylated extracellular signal-regulated kinase 1/2, excision repair cross-complementing gene 1 expression in HepG2, HepG2/oxaliplatin and HepG2/oxaliplatin/T detected by Western blotting. A-C: In comparison with in the parental HepG2 cell line, the expression levels of the fibroblast growth factor receptor 2 (FGFR2), phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and excision repair cross-complementing gene 1 (ERCC1) in the resistant cell line HepG2/oxaliplatin (OXA) were significantly increased, however, the pERK1/2 and ERCC1 expression levels in HepG2/OXA/T cells were significantly inhibited, as FGFR2 expression was silenced. The expression levels of pERK1/2 and ERCC1 in the HepG2 cells and that of FGFR2, pERK1/2 and ERCC1 in HepG2/OXA cells, was increased in the OXA + fibroblast growth factor 7 (FGF7) group and decreased in the OXA + emodin group with statistical significance compared with the OXA group, but the trend of those significant differences disappeared in HepG2/OXA/T cells ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* OXA group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* OXA + FGF7 group); D: Western blotting of FGFR2, pERK1/2, ERCC1 in HepG2, HepG2/oxaliplatin and HepG2/oxaliplatin/T. M: Marker.

demonstrates a synergistic effect and reverses platinum resistance. In the human multidrug-resistant breast cancer cell line MCF-7/Adr, the reversal index of emodin was 2.86 and 1.79 in combination with doxorubicin and cisplatin, respectively[28]. Our study results are similar, yielding a value of 3.05 in resistance reversal effect for 10 μmol/L in HepG2/OXA cells.

DNA interstrand cross-linking or chain cross-linking caused by platinum drugs induces apoptosis in tumor cells[29]. Although the capacity to repair DNA damage by NER, which removes the chain of platinum drug-induced DNA adducts, is considered to be the main mechanism of tumor cell resistance to platinum, the synergistic effect and reversal of platinum resistance induced by emodin appears to function by enhancing the DNA damage[30]. This study showed that TE, OTM and PTD were significantly higher in HepG2 cells after OXA and OXA + emodin treatment; moreover, differences are also statistically significant between the OXA group and the OXA + emodin group. A similar trend was observed in the resistant cell line HepG2/OXA. Our results suggest that emodin promotes the DNA damage that is induced by OXA. As the rate-limiting enzyme of the NER process, ERCC1 and XPF form heterodimers that exhibit damage recognition and nucleic acid cutting activity at the 5' end of the DNA in platinum-based chemotherapy. ERCC1 plays an important role in HCC, which was expected given that this gene is an effective predictor of the sensitivity of tumor cells to platinum-based chemotherapy[15,16]. This study observed that an increase in the DNA damage level occurred in the resistant cell line HepG2/OXA and their parental cell line HepG2 after being treated with emodin combined with OXA. Moreover, ERCC1 expression levels were significantly decreased. The study results suggested that emodin-mediated down-regulation of the expression of ERCC1 plays an important role in enhancing the DNA damage induced by OXA. Zhou *et al*[28] also found that ERCC1 protein expression decreases gradually in MCF-7/Adr cells in proportion to the duration of emodin treatment and that the effect of 20 μg/mL of emodin was greater than that of 10 μg/mL.

In the multidrug-resistant gastric cancer cell lines, our previous results indicated that *ERCC1* was a target gene in the FGFR2 signaling pathway[18]. The same result was observed in HCC drug-resistant cell lines in this study. Accompanied with Bek shRNA silencing of *FGFR2* gene expression, the expression of ERCC1 was significantly reduced in the drug-resistant cell line HepG2/OXA, whereas after FGF7 stimulation of the FGFR2 signaling pathway, ERCC1 expression was significantly increased. These results support the idea that ERCC1 is a downstream gene of the FGFR2 signaling pathway. FGFR2, a member of the transmembrane tyrosine kinase receptor family (FGFRs) and the expression product of the bek oncogene, plays an important role in the cell differentiation of stomach cancer[31,32] and HCC[17], which effectively predicts overall survival and progression-free survival as a molecular marker. The protein kinase C, Ras/Raf/MEK/ERK, janus kinase/signal transducer and activator of transcription, and PI3K signaling pathways are downstream cascades in the FGF-induced signaling pathways[33]. ERCC1 expression can be inhibited by the ERK inhibitor U0126, suggesting that *ERCC1* is one of the target genes in the downstream of the ERK signaling pathway[8]. Our results suggested that ERCC1 expression was inhibited by bek gene silencing; additionally, ERCC1 expression was significantly increased by the positive stimulus of FGF7. In addition, p-ERK1/2, the key molecule in the Ras/Raf/MEK/ERK pathway, was increased or reduced in conjunction with FGF7 stimulus or bek gene silencing, suggesting that the Ras/Raf/MEK/ERK pathway may be an important pathway in the FGFR2 regulation of ERCC1 expression. The most interesting result of this study was that FGFR2 protein expression disappeared accompanied with bek gene being silenced, whereas ERCC1 and p-ERK1/2 expression were not completely inhibited, suggesting that other signaling pathways may be involved in the pathway by which FGFR2 regulates ERCC1 expression.

By contrast to FGF7 stimulation of the FGFR2 signal pathway in the resistant hepatic cancer cell line HepG2/OXA and the parental cell line HepG2, the p-ERK1/2 phosphorylation level was significantly inhibited by emodin treatment; meanwhile ERCC1 expression levels were significantly decreased. These data were consistent with the results of Ko *et al*[9], who observed that emodin could significantly enhance the cytotoxicity of platinum drugs in lung cancer cell lines, and its mechanism is closely related to the inhibition of ERCC1 expression, and the downward effect of ERCC1 expression was achieved through the inactivation of the ERK1/2 pathway. Emodin, a tyrosine kinase inhibitor[19,34] and FGF7, a positive stimulator, had no effect on the expression of ERCC1 and p-ERK1/2 if FGFR2 expression was inhibited by shRNA silencing, which suggested that the emodin regulation of ERCC1 expression by the ERK1/2 pathway was closely related to the inhibition of FGFR2 tyrosine kinase activity.

In summary, the results of this study indicated that emodin could significantly enhance the DNA damage that was caused by OXA and induce OXA resistance reversal in HepG2/OXA cells. The molecular mechanism for this phenomenon is mediated by the inhibition of ERCC1 expression by the FGFR2/ERK1/2 signaling pathway.

COMMENTS

Background

As the effect of platinum-based chemotherapy on advanced hepatocellular carcinoma (HCC) was re-proved in 2010, the drugs which could strengthen chemotherapy effects and protect normal liver cells were the hot research area. Emodin, as the main active ingredient in many Chinese herbs, interested us for its low toxicity and synergistic effect combined with platinum in HCC cells. Excision repair cross-complementing gene 1 (*ERCC1*), which was the limiting enzyme in the nucleotide excision repair pathway, plays an important role in the process of platinum drug resistance. The relationship between synergistic effects and the regulation of ERCC1 expression is worthy of further study in HCC treatments that combine emodin with platinum drugs.

Research frontiers

Fibroblast growth factor receptor 2 (FGFR2), as a transmembrane tyrosine kinase, plays an important role in the differentiation of HCC, the clinical staging of tumors, the incidence of tumor thrombosis and the determination of alpha-fetoprotein levels. Interestingly, ERCC1 is a downstream target gene of *FGFR2*, whereas emodin is a tyrosine kinase inhibitor. Current research has indicated that emodin down-regulates ERCC1 expression in non-small cell lung cancer, and its effects may be relevant to the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, which is one of the downstream components of the FGFR2 pathway. However, the exact mechanism through which emodin produces these effects has not been well established.

Innovations and breakthroughs

In this study, the resistance reversal effect for 10 μmol/L emodin was 3.05 in HepG2/oxaliplatin (OXA) cells. Meanwhile, the tail length, the olive tail length and the percentage of tail DNA were significantly higher after treatment combined with emodin, which suggest that emodin promotes the DNA damage induced by OXA. Accompanied with bek shRNA silencing and fibroblast growth factor 7 (FGF7) stimulation of *FGFR2* gene expression, the expression of phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and ERCC1 was significantly reduced and increased in the drug-resistant cell line HepG2/OXA, which supports the idea that *ERCC1* is a downstream gene of the FGFR2/p-ERK1/2 signaling pathway. Furthermore, ERCC1 expression levels in HepG2/OXA and HepG2 cells were significantly decreased after emodin treatment with significant inhibition of the p-ERK1/2 phosphorylation level. However, if FGFR2 expression was inhibited by shRNA silencing, the inhibition effect of emodin and the stimulation effect of FGF7 on the expression of ERCC1 and p-ERK1/2 disappeared, which suggested that emodin regulation of ERCC1 expression by the ERK1/2 pathway was closely related to the inhibition of FGFR2 tyrosine kinase activity.

Applications

The results of emodin enhancing the DNA damage caused by OXA and its molecular mechanism associated with the inhibition of ERCC1 expression by the FGFR2/ERK1/2 signaling pathway may provide an experimental basis for the further development and application of emodin in the reversal of platinum drug resistance in HCC and other types of malignant tumors.

Terminology

Platinum drug resistance: Platinum-based compounds, including cisplatin, carboplatin and OXA, are widely used in a number of carcinomas, and compose a mainstay of chemotherapeutic treatment. The cytotoxicity of platinum is attributed to apoptosis induced by DNA damage through the formation of platinum crosslinks on DNA. Cancer cells have the capacity to decrease the platinum concentration and repair DNA damage, which is associated with platinum drug resistance. The capacity to remove the chain of platinum drug-induced DNA adducts, is considered to be the main mechanism of tumor cell resistance to platinum.

Peer review

This is a good descriptive study in which authors proved emodin could significantly enhance the DNA damage caused by OXA and induce OXA resistance reversal in HepG2/OXA cells, and investigated the molecular mechanism for this effect from the point of the inhibition of ERCC1 expression mediated by the FGFR2/ERK1/2 signaling pathway. The results are interesting and suggest that emodin is a potential therapeutic substance that could be used in reversing platinum drug resistance in the HCC and other types of malignant tumors.

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ORIGINAL ARTICLE

Correlation of fibrinogen-like protein 2 with progression of acute pancreatitis in rats

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Abstract

AIM: To examine fibrinogen-like protein 2 (fgl2) expression during taurocholate-induced acute pancreatitis progression in rats and its correlation with pancreatic injury severity.

METHODS: Forty-eight male Sprague-Dawley rats were randomly divided into the severe acute pancreatitis (SAP) group (n = 24) and the sham operation (SO) group (n = 24). Sodium taurocholate (4% at doses of 1 mL/kg body weight) was retrogradely injected into the biliopancreatic ducts of the rats to induce SAP. Pancreatic tissues were prepared immediately after sacrifice. At the time of sacrifice, blood was obtained for determination of serum amylase activity and isolation of peripheral blood mononuclear cells (PBMCs). Pancreatic tissue specimens were obtained for routine light microscopy including hematoxylin and eosin staining, and the severity of pancreatic injury was evaluated 1, 4 and 8 h after induction. Expression of fgl2 mRNA was measured in the pancreas and PBMCs using reverse transcription polymerase chain reaction. Expression of fgl2 protein was evaluated in pancreatic tissues using Western blotting and immunohistochemical staining. Masson staining was also performed to observe microthrombosis.

RESULTS: At each time point, levels of fgl2 mRNAs in pancreatic tissues and PBMCs were higher ($P < 0.05$) in the SAP group than in the SO group. For pancreatic tissue in SAP *vs* SO, the levels were: after 1 h, 3.911 ± 1.277 *vs* 1.000 ± 0.673; after 4 h, 9.850 ± 3.095 *vs* 1.136 ± 0.609; and after 8 h, 12.870 ± 3.046 *vs* 1.177 ± 0.458. For PBMCs in SAP *vs* SO, the levels were: after 1 h, 2.678 ± 1.509 *vs* 1.000 ± 0.965; after 4 h, 6.922 ± 1.984 *vs* 1.051 ± 0.781; and after 8 h, 13.533 ± 6.575 *vs* 1.306 ± 1.179. Levels of fgl2 protein expression as determined by Western blotting and immunohistochemical staining were markedly up-regulated ($P < 0.001$) in the SAP group compared with those in the SO group. For Western blotting in SAP *vs* SO, the results were: after 1 h, 2.183 ± 0.115 *vs* 1.110 ± 0.158; after 4 h, 2.697 ± 0.090 *vs* 0.947 ± 0.361; and after 8 h, 3.258 ± 0.094 *vs* 1.208 ± 0.082. For immunohistochemical staining in SAP *vs* SO, the results were: after 1 h, 1.793 ± 0.463 *vs* 0.808 ± 0.252; after 4 h, 4.535 ± 0.550 *vs* 0.871 ± 0.318; and after 8 h, 6.071 ± 0.941 *vs* 1.020 ± 0.406. Moreover, we observed a positive correlation in the pancreas ($r = 0.852$, $P < 0.001$) and PBMCs ($r = 0.735$, $P < 0.001$) between fgl2 expression and the severity of pancreatic injury. Masson staining showed that microthrombosis (%) in rats with SAP was increased ($P < 0.001$) compared with that in the SO group and it was closely correlated with fgl2 expression in the pancreas ($r = 0.842$, $P < 0.001$). For Masson staining in SAP *vs* SO, the results were: after 1 h, 26.880 ± 9.031 *vs* 8.630 ± 3.739; after 4 h, 53.750 ± 19.039 *vs* 8.500 ± 4.472; and after 8 h, 80.250 ± 12.915 *vs* 10.630 ± 7.003.

CONCLUSION: Microthrombosis due to fgl2 overexpression contributes to pancreatic impairment in rats with SAP, and fgl2 level may serve as a biomarker during early stages of disease.

Key words: Fibrinogen-like protein 2; Microthrombosis; Fibrin; Severe acute pancreatitis; Peripheral blood mononuclear cell

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INTRODUCTION

Severe acute pancreatitis (SAP) is a pathogenic condition that progresses rapidly and has a high mortality[1-3], but the underlying pathophysiological mechanisms remain incompletely defined. SAP is currently considered to be complicated by microcirculatory disturbances and coagulation abnormalities[4,5]. Inflammatory mediators such as interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α) released during acute inflammatory reactions are not just involved in the inflammatory process but may also be responsible for the systemic activation of hemostasis in patients with SAP[6,7]. Intravascular coagulation and thromboembolism are believed to play an important role in the pathogenesis of SAP and are related to its severity[8,9]. Acute inflammatory events during disease progression can lead to dysregulation of the coagulation cascade[10]. In SAP patients, thrombin and platelets are deposited not only in the local pancreatic blood vessels but also in the connective tissue and intercellular spaces[4]. Studies suggest that biochemical variables such as prothrombin time, D-dimer, and clotting time may have prognostic value, and direct anticoagulant therapy has been shown to be helpful in the treatment of SAP[4,10-12]. These facts suggest that coagulation and inflammation in SAP are correlated, thus microthrombosis plays a crucial role in SAP[10]. However, the exact pathophysiological mechanism remains unknown.

Fibrinogen-like protein 2 (fgl2)/fibroleukin (also termed fgl2 prothrombinase) was determined to be a new member of the fibrinogen-related protein superfamily (fibrinogen-related domain), which includes fibrinogen, tenascin, ficolin, and angiopoietin[13-15]. fgl2 is a direct prothrombinase with serine protease activity. fgl2 can cleave prothrombin to thrombin *via* a noncanonical pathway, resulting in fibrin deposition[16,17]. fgl2 leads to histopathological lesions and ischemic injury by mediating "immune coagulation", fibrin deposition, and microthrombosis[18-21]. Microvascular disturbances are caused by microthrombi that are activated and produced as a consequence of fgl2 action[18,21-23]. Nevertheless, whether fgl2 contributes to the pathogenesis of SAP is unclear.

In the present study, we used 4% sodium taurocholate to induce SAP in rats. We then investigated the expression and localization of fgl2 in pancreatic tissues. We also assessed fgl2 expression and its correlation with severity of pancreatic injury and microthrombi in rats with SAP to provide new insight into the pathogenesis of this disease.

MATERIALS AND METHODS

Animals

Forty-eight male Sprague-Dawley rats, weighing 200-250 g, were obtained from the Experimental Animal Center of Wenzhou Medical College, Wenzhou, China. All animals were fed standard rat chow, had free access to water, and were housed at a constant room temperature of 25 ℃ and a 12-h day/night cycle. All animals were acclimated for at least one week before the experiments were initiated. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical College.

Induction of SAP

All rats received intraperitoneal injection of 10% chloraldurate (2 mL/kg body weight; Solarbio, Beijing, China) for anesthesia. The rats were divided into the SAP group (n = 24) and the sham operation (SO) group (n = 24). In the SAP group, a laparotomy was performed through a midline incision. Sodium taurocholate (4%; 1 mL/kg body weight; Sigma, St. Louis, MO, United States) was retrogradely injected into the biliopancreatic duct through the papilla using a segmental epidural catheter *via* a microinjection pump at a speed of 0.2 mL/min. A microclip was placed in the hepatic portion of the biliopancreatic duct to avoid reflux before the injection. SO rats underwent surgery but without infusion. After each operation, the abdomen was closed in two layers. All procedures were carried out using sterile techniques.

Sample collection and determination of serum amylase

At defined time points (1, 4 and 8 h; n = 8 per time point) after SAP induction, rats were anesthetized with 10% chloraldurate (2 mL/kg body weight) and euthanized by exsanguination. Pancreatic tissues were harvested immediately and divided into two pieces. Portions of the tissues were fixed in 4% paraformaldehyde for immunohistochemical staining and microscopic observation, and other portions were removed and stored in liquid nitrogen until use. Blood samples (5 mL) were obtained *via* postcava puncture, and 4 mL of each sample was collected and stored in 5-mL tubes without anticoagulants (Generay, Shanghai, China). The blood samples were centrifuged at 1200 × g for 20 min, and the serum was collected for determination of amylase activity (U/L) with a fully automatic biochemical analyzer (Hitachi, Tokyo, Japan). The remaining 1 mL blood was stored in

ethylene diamine tetraacetic acid-containing tubes (Generay) and used to isolate peripheral blood mononuclear cells (PBMCs).

Isolation of PBMCs

PBMC isolation was performed with density gradient centrifugation. The blood sample (1 mL) was diluted with 1 mL 0.9% saline. Subsequently, the diluted cell suspension was carefully laid over 2 mL Bandicoot percoll (Solarbio) and centrifuged at 2000 × *g* for 20 min at 20 ℃. The PBMC layer was carefully transferred into a new tube, and the volume was brought to 5 mL with 0.9% saline and centrifuged (2000 × *g*, 5 min, 20 ℃). This step was repeated. Finally, the supernatant was carefully removed, reserving the PBMCs at the bottom of the tube. PBMCs were immediately stored in liquid nitrogen until use.

Histological analysis and assessment of pancreatic tissue injury

Pancreatic tissue samples were fixed in 4% paraformaldehyde for histological analysis. The samples were dehydrated and embedded in paraffin. Pancreas sections were stained with hematoxylin and eosin (HE) for routine light microscopy. Slides were examined in a blinded fashion by two pathologists who were unaware of the treatment protocol according to the modified method of Schmidt *et al*[24] and Eşrefoğlu *et al*[25]. Variables of edema, hemorrhage, acinar cell degeneration, and interstitial inflammation were scored in 10 random fields of each slide to assess the severity of pancreatic injury under a light microscope (CX31, Tokyo, Japan; HE staining, × 200). Each variable was scored as follows: (1) edema: 0 = absent, 1 = focally in the interlobular space, 2 = increased in the intralobular space, 3 = isolated-island appearance of pancreatic acinus; (2) hemorrhage: 0 = absent, 1 = slight, 2 = moderate, 3 = severe; (3) acinar cell degeneration: 0 = absent, 1 = focal (< 5%); 2 = and/or sublobular (< 20%), 3 = and/or lobular (> 20%); and (4) inflammation: 0 = absent, 1 = slight, 2 = moderate, 3 = severe. The sum of these four variables for each pancreas section could have a maximum score of 12.

Pancreatic tissue sections were also stained with Masson stain to observe microthrombi in microvessels. One hundred microvessels on each slide were randomly selected, and the percent of microthrombus-positive vessels was calculated.

Real-time fluorescence-based quantitative polymerase chain reaction

Total RNA was extracted from pancreatic tissues and PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA, United States), and cDNA was synthesized with the First Strand cDNA synthesis kit (MBI Fermentas, Burlington, Canada) according to the manufacturer's protocols. The samples were subsequently amplified using Moloney murine leukemia virus reverse transcriptase and Taq DNA polymerase (Invitrogen) using an ABI 7500 Sequence Detection System (Applied Biosystems Inc., Carlsbad, CA, United States). The sequences of the primers (Generay) were as follows: fgl2 (153 bp): 5'-CCTGGAGATTGTGGTTTCGT-3' (forward) and 5'-TACCATGCCTTTCTCCAAGG-3' (reverse), β-actin (153 bp): 5'-TGTCACCAACTGGGACGATA-3' (forward) and 5'-GGGGTGTTGAAGGTCTCAAA-3' (reverse). The cDNA was denatured at 95 ℃ for 5 min and amplified for 40 cycles of 95 ℃ (15 s), 60 ℃ (45 s), and 72 ℃ (60 s), followed by a final extension at 72 ℃ (5 min). The samples were tested in triplicate, and the results were calculated using the $2^{-\Delta\Delta CT}$ method. The expression of fgl2 mRNA was shown as relative to β-actin.

Western blotting

Pancreatic proteins were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, United States). The membrane was blocked with 5% skim milk in Tris-buffered saline and then incubated with a polyclonal antibody against fgl2 (Biosynthesis Biotechnology, Beijing, China; 1:200) at 4 ℃ overnight. The membrane was washed three times with Tris-buffered saline and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) conjugated to horseradish peroxidase for 2 h at room temperature. The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, United States). Protein expression levels were normalized to β-actin.

Immunohistochemical staining

Immunohistochemical staining was performed to assess fgl2 protein expression in the pancreas using EnVision reagents (Dako, Glostrup, Denmark). Four-μm-thick paraffin sections were routinely cut. Microwave antigen retrieval was conducted for 20 min in citrate buffer (pH 6.0) to activate antigens before quenching endogenous peroxidase activity in 0.3% H_2O_2 for 10 min. After three times of washing with phosphate-buffered saline (Generay) and then the primary reaction solution, the sections were incubated with rabbit polyclonal anti-rat fgl2 (Biosynthesis Biotechnology; 1:100) for 2 h at 37 ℃. Following the same washing procedure, EnVision reagents were applied and incubated for 30 min at 37 ℃. Finally, the reaction was developed with 0.05% diaminobenzidine and counterstained with hematoxylin for microscopy. Phosphate-buffered saline was used as a negative control instead of the primary antibody. To measure fgl2 protein expression, 10 randomly selected fields across each section were evaluated at × 200 magnification.

Statistical analysis

All data represent the mean ± SD. SPSS 15.0 software (SPSS, Chicago, IL, United States) was used for statistical analysis. Differences between the SAP and SO groups were analyzed with the Student's *t* test. One-way analysis of variance was used to check for statistical significance

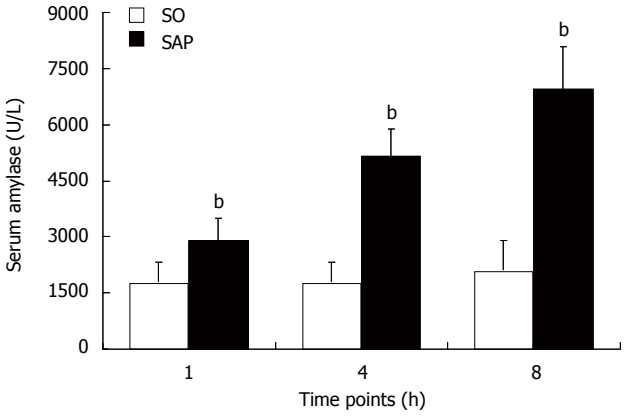


Figure 1 Levels of serum amylase in severe acute pancreatitis and sham-operated rats at each time point. Each time point (h) after operation consisted of 8 rats. There was no difference among the three time points in the sham operation (SO) group ($P > 0.05$). The data are expressed as the mean ± SD. $^{b}P < 0.01$ *vs* SO group. SAP: Severe acute pancreatitis.

among the three time points in the same group. Pearson's correlation coefficient was calculated to determine the strength of the association between two continuous variables. $P < 0.05$ was considered statistically significant.

RESULTS

Levels of serum amylase are elevated in rats with SAP

Serum amylase is the most commonly used biochemical indicator of acute pancreatitis. Levels of serum amylase were markedly elevated ($P < 0.01$) in the SAP group compared with the SO group at each time point. There was no significant difference in the levels of serum amylase among the three time points in the SO group (Figure 1).

Histopathology and pathological scoring of pancreatic tissues

Compared with the SO group, the pancreatic tissues in the SAP group (Figure 2A-C) at 1 h (A), 4 h (B), and 8 h (C) appeared much more severely damaged. The pancreatic tissue of the SO group (Figure 2D) appeared morphologically normal at 8 h. Microscopic examination of the pancreas in the SAP group showed edema, hemorrhage complicated by microthrombosis, acinar cell degeneration, and inflammation (Figure 2A-C). The mean pathological score of each rat with SAP was higher ($P < 0.01$) than that of control rats at each time point. Pancreatitis worsened over time, as demonstrated by the increasing pathological score ($P < 0.01$, Figure 2E).

Masson staining was used to observe microthrombosis, which was seen as very bright red regions under light microscope. Microthrombi were localized and tightly combined with the microvascular endothelium of the pancreatic tissues, which suggested that the microthrombi formed *in situ* with the formation of fibrin. The percent of Masson staining-positive microvessels in the pancreas of the SAP group was higher ($P < 0.01$) than that in control rats at all time points and tended to increase ($P < 0.01$, Figure 3).

Up-regulation of fgl2 mRNA and protein expression in rats with SAP

fgl2 expression was evaluated with real-time polymerase chain reaction, Western blotting, and immunohistochemical staining. The level of fgl2 mRNA was elevated in both pancreatic tissues and PBMCs ($P < 0.05$) beginning at 1 h after injection of 4% sodium taurocholate compared to the SO group. fgl2 mRNA increased over time in the SAP group ($P < 0.01$, Figure 4A and B). Western blot analysis revealed that fgl2 protein expression in the pancreas was higher ($P < 0.01$) in the SAP group than in the SO group and showed a tendency to increase over time ($P < 0.01$, Figure 4C and D). Immunohistochemical staining demonstrated that fgl2 was strongly expressed and localized in microvascular endothelial cells in pancreatic tissues in rats with SAP (Figure 5A-C). Only low levels of fgl2 expression were found in control rats (Figure 5D). In accordance with fgl2 mRNA level, fgl2 protein level was elevated as indicated by the mean absorbance value ($P < 0.01$) in the SAP group compared to the control rats and tended to increase ($P < 0.01$, Figure 5E). Pearson's correlation coefficient analysis was used to compare fgl2 expression and the proportion of Masson staining-positive microvessels, and the results showed a correlation ($r = 0.842$, $P < 0.01$). This result suggested that elevated fgl2 expression may contribute to microthrombosis.

fgl2 expression is relevant to the severity of pancreatic injury in rats with SAP

fgl2 expression and the severity of pancreatic injury of rats with SAP (as indicated by the pathological score) were higher compared with control rats, indicating a correlation between fgl2 expression and disease severity upon induction of SAP. Moreover, fgl2 expression in the pancreas ($r = 0.852$, $P < 0.01$) and PBMCs ($r = 0.735$, $P < 0.01$) correlated with the severity of pancreatic injury.

DISCUSSION

SAP is an inflammatory disorder mediated by up-regulated expression of proinflammatory cytokines such as TNF-α[4,10]. Inflammation and coagulation are interactive events during SAP. Microthrombosis is found in the early stages of the SAP rat model[1]. The "immune coagulation" hypothesized by Levy means that fgl2 could be transcribed and the mRNA translated following the induction of cytokines such as IL-2 and TNF-α, resulting in immediate activation of coagulation[17,23,26,27]. fgl2 functions as a bridge molecule between immune and coagulation reactions. fgl2 is highly expressed in endothelial cells due to the action of TNF-α[27,28]. Otherwise, interferon-γ is necessary for macrophage induction of fgl2[27]. In the present study, fgl2 was clearly up-regulated and localized in inflammatory regions of the pancreas sections, suggesting that fgl2 as an effector molecule may contribute to SAP pathogenesis by initiating and promoting coagulation through the induction of proinflammatory cytokines such as TNF-α.

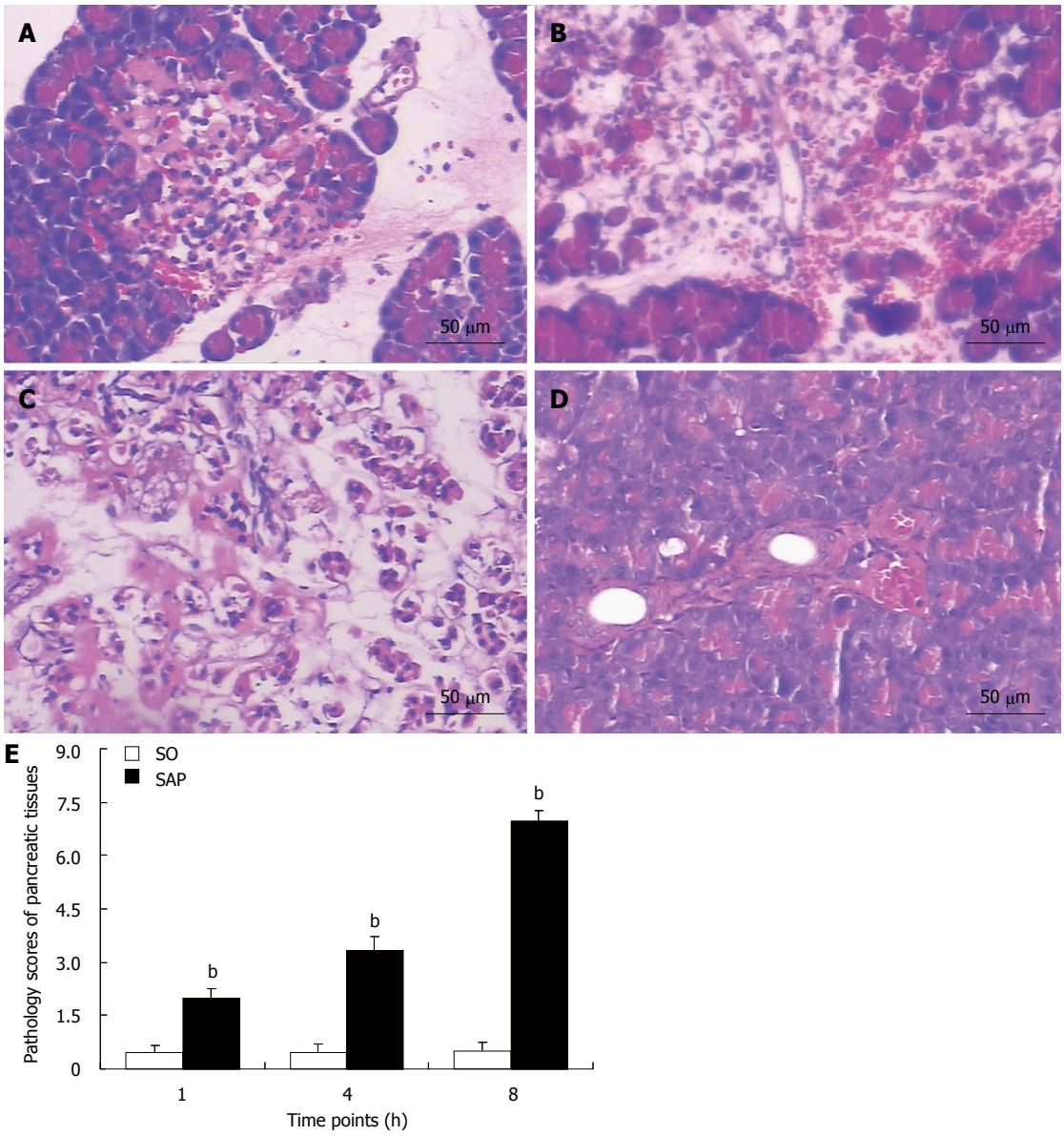


Figure 2 Histology of pancreatic tissues and pathological scores of pancreatic tissues from severe acute pancreatitis and sham-operated rats. A-C: Histological changes in pancreatic tissues at 1 h (A), 4 h (B), and 8 h (C) in the severe acute pancreatitis (SAP) group; D: Histological changes in pancreatic tissues at 8 h in the sham operation (SO) group (hematoxylin and eosin staining; × 200); E: Pathological scores of pancreatic tissues. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean ± SD. $^{b}P < 0.01$ *vs* SO group.

fgl2/fibroleukin is a new procoagulant that belongs to the fibrinogen-related protein superfamily, which has a potent capability of inducing microthrombosis[16,17,29]. fgl2 is expressed in activated macrophages, T cells, and endothelial cells[30]. fgl2 expression and the subsequent fibrin deposition account for microthrombus formation *in situ*[18], which occurs *via* a novel way by directly producing thrombin in addition to the classic extrinsic and intrinsic coagulant pathway[16,17]. Researches suggest that microcirculatory disturbance is an important aspect of the mechanism of SAP[4,8]. Our data show that microthrombi generated during pancreatitis (due to increased fgl2 expression) led to ischemia/hemorrhage injury and consequently resulted in necrosis and dysfunction of the pancreas. Moreover, we found that fgl2 plays a contributing role in pancreatic microthrombus formation in rats with SAP. Our study also shows that fgl2 has procoagulant activity in the pancreatic endothelial cells of microvessels in rats with SAP, and fgl2 expression correlates strongly with the severity of pancreatic injury.

We observed that both fgl2 mRNA and protein levels were higher in rats with SAP and that the levels gradually increased in parallel with the progression of SAP. We also observed that fgl2 expression was associated with microthrombus formation and that microthrombus formation *in situ* may be caused by fgl2, leading to partial impairment of the pancreatic tissues and the functions involved. We propose that fgl2 functions similarly as in other diseases[26,31-33]: microthrombi form as a consequence of fgl2 expression in the pancreas, leading to microcirculatory disturbance and consequent hemorrhage/ischemia injury in rats with SAP, thus aggravating the pancreatic injury.

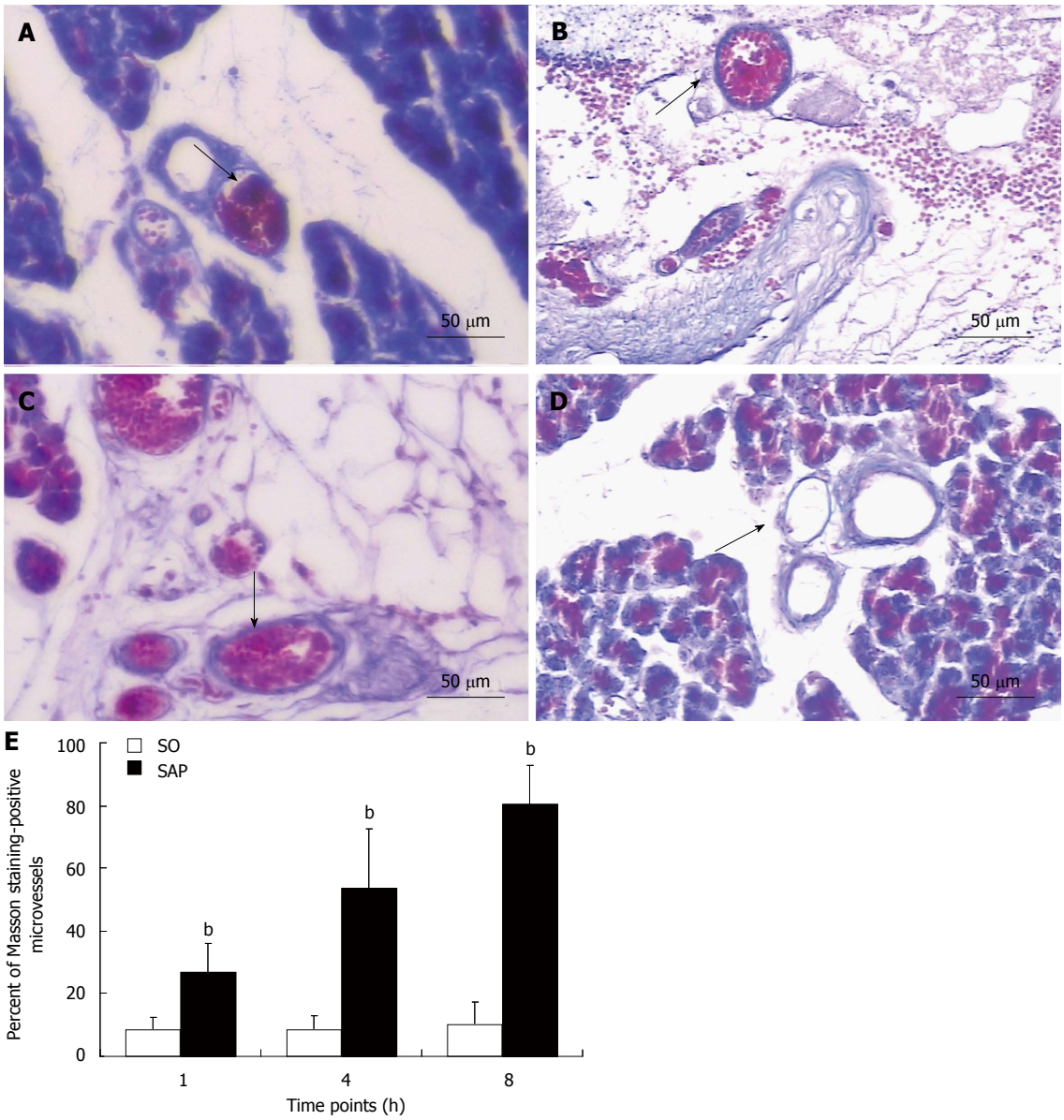
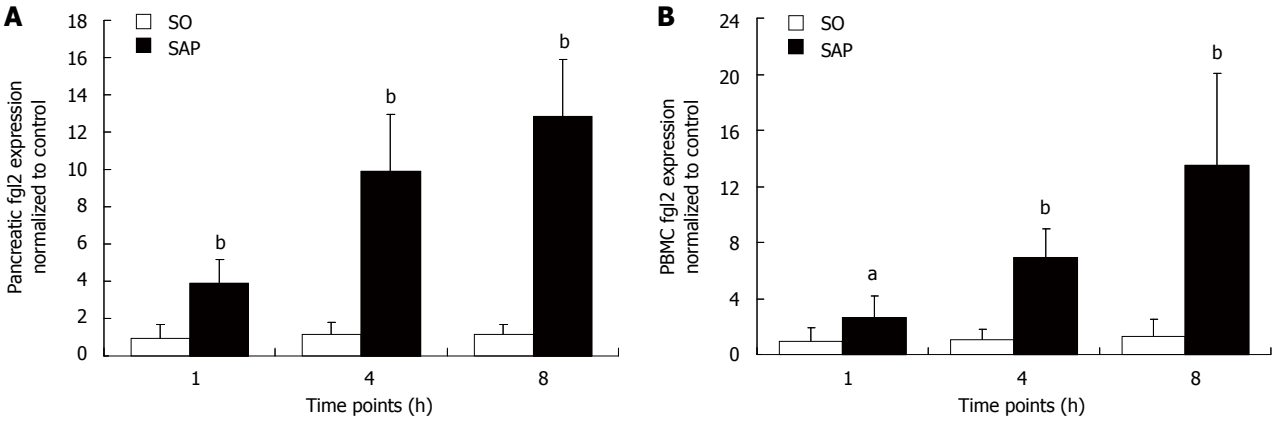


Figure 3 Masson staining of microthrombosis in pancreatic microvessels from severe acute pancreatitis and sham-operated rats (× 200). A-C: Microthrombosis *in situ* in pancreatic microvessels of rats with severe acute pancreatitis (SAP) (arrows) at 1 h (A), 4 h (B), and 8 h (C) in the SAP group; D: No microthrombi were detected in the sham operation (SO) group (arrow); E: The percent of Masson staining-positive microvessels. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean ± SD. $^{b}P < 0.01$ *vs* SO group.



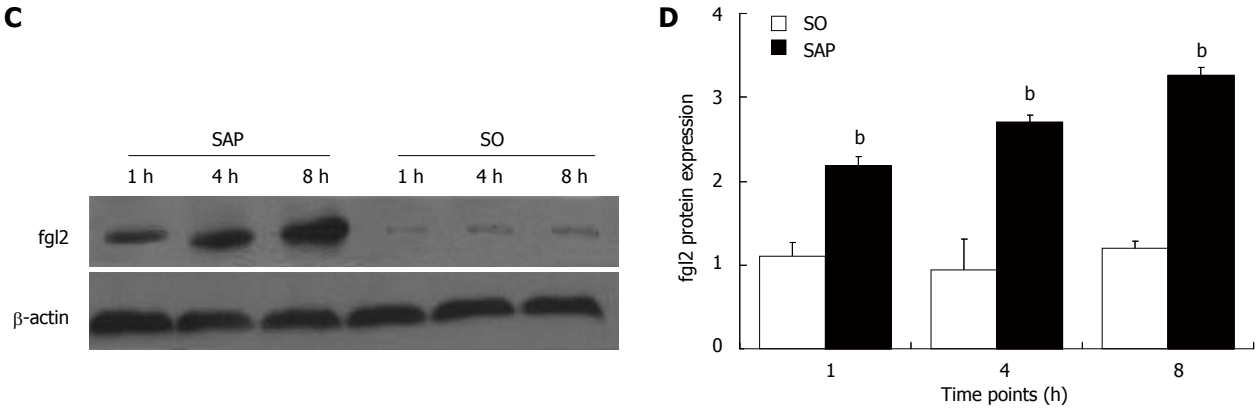


Figure 4 Levels of fibrinogen-like protein 2 mRNA and protein in severe acute pancreatitis and sham-operated rats. A, B: The calculated levels of fibrinogen-like protein 2 (fgl2) mRNA in the pancreas and peripheral blood mononuclear cells (PBMCs). The expression of fgl2 mRNA is relative to β-actin; C, D: fgl2 protein expression revealed by Western blotting. Protein levels were normalized to β-actin. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* sham operation (SO) group. SAP: Severe acute pancreatitis.

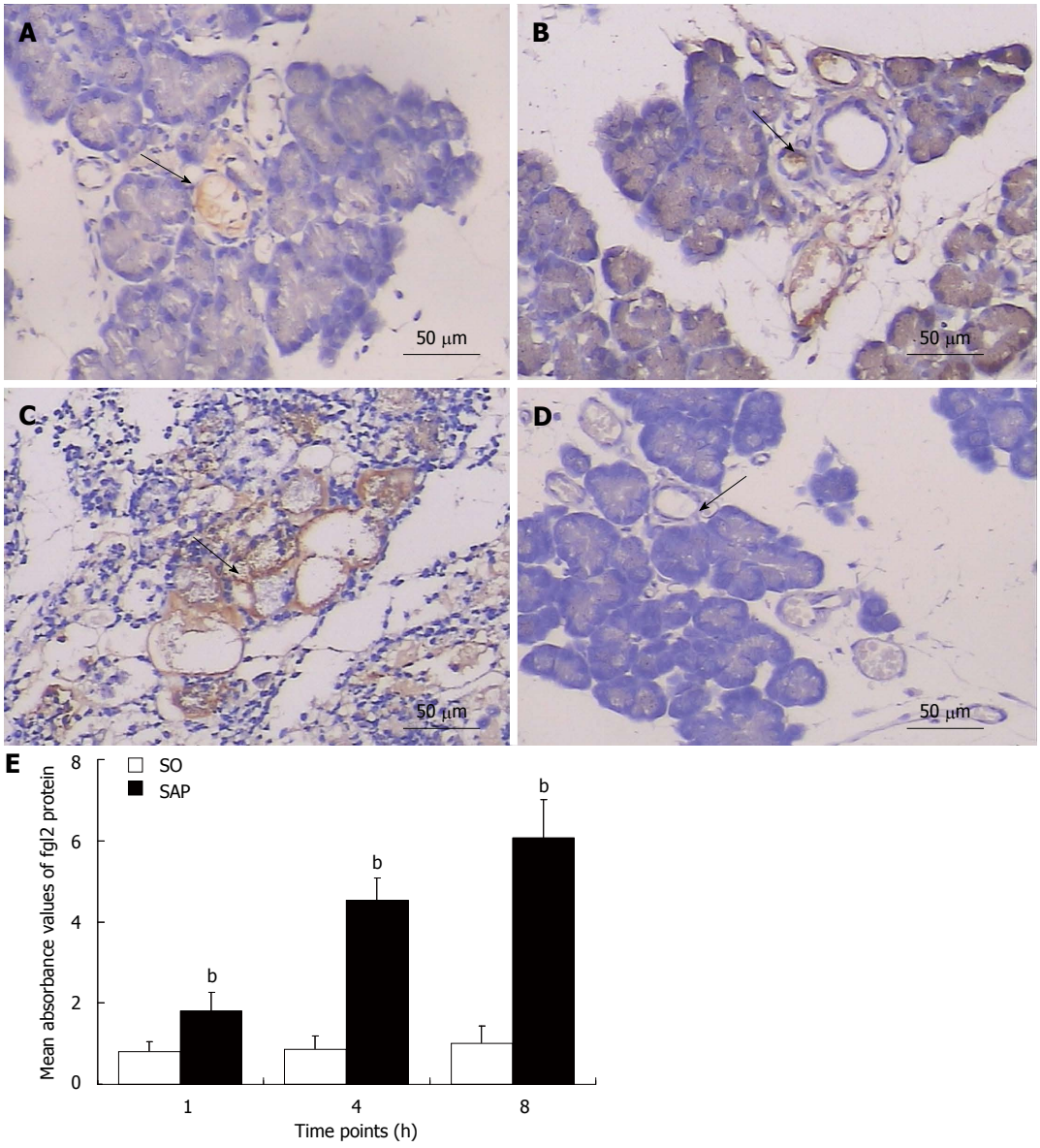


Figure 5 Fibrinogen-like protein 2 expression in pancreatic tissues of severe acute pancreatitis and sham-operated rats with immunohistochemical staining (× 200). A-D: Fibrinogen-like protein 2 (fgl2) proteins were expressed in microvascular endothelial cells of rats with severe acute pancreatitis (SAP) (arrows in A-C) and control rats (arrow in D) at 1 h (A), 4 h (B), and 8 h (C, D) after initiation of SAP; E: fgl2 protein expression is indicated by the mean absorbance value. The data are expressed as the mean ± SD. $^{b}P < 0.01$ *vs* sham operation (SO) group.

To evaluate the relevance of fgl2 expression and the severity of pancreatic disease, a Pearson's correlation coefficient was calculated. Evaluation of fgl2 expression levels in both the pancreas and PBMCs (containing lymphocytes, monocytes, dendritic cells, and other cell types) revealed a strong correlation with the severity of pancreatic disease as illustrated by the pathological score. Thus, fgl2 expression may serve as a promising marker for predicting the occurrence of SAP in early stages.

Injection of a neutralizing antibody or genetic therapy against fgl2 in diseases involving fgl2, such as murine hepatitis virus 3-induced hepatitis and graft rejection, has been beneficial in terms of attenuating fibrin deposition and pathology and preventing death in mice[18,34-36]. Thus, we will perform an in-depth investigation to see whether inhibiting fgl2 activity or applying fgl2 antibodies will delay or ameliorate the disease course of SAP.

In summary, fgl2 functions as a novel prothrombinase and may initiate the coagulation reaction, finally leading to microthrombosis in microvessels of pancreatic tissues in an experimental model of rats with SAP, and resulting in ischemia/hemorrhage injury as well as necrosis and dysfunction of the pancreas. fgl2 expression is closely correlated with the severity of pancreatic disease, and thus fgl2 may serve as a useful biomarker for predicting SAP at the onset of disease. Whether inhibiting fgl2 or using antibodies against fgl2 will delay or ameliorate SAP requires further exploration.

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COMMENTS

Background

Fibrinogen-like protein 2 (fgl2)/fibroleukin (also termed fgl2 prothrombinase) is a recently discovered member of the fibrinogen-related protein superfamily. fgl2 is a direct prothrombinase with serine protease activity and can cleave prothrombin to thrombin *via* a noncanonical pathway, resulting in fibrin deposition. Several studies have demonstrated that fgl2 leads to pathology by mediating "immune coagulation", fibrin deposition, and microthrombosis in murine hepatitis virus 3-induced fulminant hepatitis, spontaneous abortion, xenograft rejection, and type 2 diabetic nephropathy.

Research frontiers

Intravascular coagulation and thromboembolism play a pivotal role in the pathogenesis of severe acute pancreatitis (SAP) and are related to its severity. Acute inflammatory events during SAP may result in dysregulation of the coagulation cascade. However, whether fgl2 is involved in the pathogenesis of SAP has not been studied. In this study, the authors demonstrate that increased expression of fgl2 contributes to pancreatic impairment in rats with SAP by mediating microthrombosis. Thus, fgl2 level may serve as a useful biomarker at early stages of disease.

Innovations and breakthroughs

The mechanism(s) responsible for the microcirculatory disturbances and coagulation abnormalities during the SAP process remains to be elucidated. This is the first study to report that fgl2 is highly expressed in microvascular endothelial cells of pancreatic tissues in rats with SAP. Furthermore, microthrombosis due to flg2 contributes to pancreatic impairment in rats with SAP, and fgl2 level may serve as a useful biomarker at disease onset.

Applications

In the present study, the authors investigated fgl2 expression and localization in the pancreas of rats with SAP, which will provide new insight into the pathogenesis of SAP and efficacious anticoagulant therapy for SAP treatment.

Terminology

SAP is principally caused by autodigestion of the pancreas and is a potentially fatal pathogenic condition characterized by rapid progression and high mortality. fgl2 is a new member of the fibrinogen-related protein superfamily, which includes fibrinogen, tenascin, ficolin, and angiopoietin.

Peer review

The authors of this study investigated the pathogenesis of acute pancreatitis. Their results provide insight into the contribution of microthrombosis to the development of pathological changes during the progression of acute pancreatitis. The authors have demonstrated increased expression of fgl2 mRNA in the pancreas and peripheral blood mononuclear cells with a subsequent increase in the levels of the corresponding proteins. The anticipated pathological changes were further substantiated by Masson staining. Stringency of the proposed hypothesis was validated, revealing a strong correlation between induced coagulation disorders and pathological changes in pancreatic tissues. It is an interesting subject, and the results are clearly described.

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BRIEF ARTICLE

How do we manage post-OLT redundant bile duct?

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Abstract

AIM: To address endoscopic outcomes of post-Orthotopic liver transplantation (OLT) patients diagnosed with a "redundant bile duct" (RBD).

METHODS: Medical records of patients who underwent OLT at the Liver Transplant Center, University Texas Health Science Center at San Antonio Texas were retrospectively analyzed. Patients with suspected biliary tract complications (BTC) underwent endoscopic retrograde cholangiopancreatography (ERCP). All ERCP were performed by experienced biliary endoscopist. RBD was defined as a looped, sigmoid-shaped bile duct on cholangiogram with associated cholestatic liver biomarkers. Patients with biliary T-tube placement, biliary anastomotic strictures, bile leaks, bile-duct stones-sludge and suspected sphincter of oddi dysfunction were excluded. Therapy included single or multiple biliary stents with or without sphincterotomy. The incidence of RBD, the number of ERCP corrective sessions, and the type of endoscopic interventions were recorded. Successful response to endoscopic therapy was defined as resolution of RBD with normalization of associated cholestasis. Laboratory data and pertinent radiographic imaging noted included the pre-ERCP period and a follow up period of 6-12 mo after the last ERCP intervention.

RESULTS: One thousand two hundred and eighty-two patient records who received OLT from 1992 through 2011 were reviewed. Two hundred and twenty-four patients underwent ERCP for suspected BTC. RBD was reported in each of the initial cholangiograms. Twenty-one out of 1282 (1.6%) were identified as having RBD. There were 12 men and 9 women, average age of 59.6 years. Primary indication for ERCP was cholestatic pattern of liver associated biomarkers. Nineteen out of 21 patients underwent endoscopic therapy and 2/21 required immediate surgical intervention. In the endoscopically managed group: 65 ERCP procedures were performed with an average of 3.4 per patient and 1.1 stent per session. Fifteen out of 19 (78.9%) patients were successfully managed with biliary stenting. All stents were plastic. Selection of stent size and length were based upon endoscopist preference. Stent size ranged from 7 to 11.5 Fr (average stent size 10 Fr); Stent length ranged from 6 to 15 cm (average length 9 cm). Concurrent biliary sphincterotomy was performed in 10/19 patients. Single ERCP session was sufficient in 6/15 (40.0%) patients, whereas 4/15 (26.7%) patients needed two ERCP sessions and 5/15 (33.3%) patients required more than two (average of 5.4 ERCP procedures). Single biliary stent was sufficient in 5 patients; the remaining patients required an average of 4.9 stents. Four out of 19 (21.1%) patients failed endotherapy (lack of resolution of RBD and recurrent cholestasis in the absence of biliary stent) and required either choledocojejunostomy (2/4) or percutaneous biliary drainage (2/4). Endoscopic complications included: 2/65 (3%) post-ERCP pancreatitis and 2/10 (20%)

non-complicated post-sphincterotomy bleeding. No endoscopic related mortality was found. The medical records of the 15 successful endoscopically managed patients were reviewed for a period of one year after removal of all biliary stents. Eleven patients had continued resolution of cholestatic biomarkers (73%). One patient had recurrent hepatitis C, 2 patients suffered septic shock which was not associated with ERCP and 1 patient was transferred care to an outside provider and records were not available for our review.

CONCLUSION: Although surgical biliary reconstruction techniques have improved, RBD represents a post-OLT complication. This entity is rare however, endoscopic management of RBD represents a reasonable initial approach.

Key words: Redundant bile duct; Orthotopic liver transplantation; Biliary complications; Biliary stent; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Despite the dramatic improvements in surgical techniques, biliary tract complications (BTC) are still a significant source of morbidity and mortality after orthotopic liver transplantation (OLT)[1,2]. Since the beginning of liver transplantation, the biliary reconstruction has been a sensitive area regarding graft and recipient complications.

Presently, clinical evidence supports the choledocholedocojejunostomy over the T-tube stent placement or Roux-en-Y choledocho-jejunostomy, as the preferred method of biliary reconstruction[3,4]. It is postulated that several factors (*e.g.*, donor and recipient biliary ductal anatomy, duct-duct anastomosis technique, and blood supply to the bile ducts) can affect the final post-surgical bile duct configuration and may result in its ultimate successful function[5,6]. Surgical management used to represent the initial standard of care for BTC; however, the advancement in endoscopic therapeutic interventions has replaced prompt surgical intervention in most of the immediate and delayed complications[7-12].

Endoscopic therapy has been successful in the management of BTC. During the performance of the endoscopic retrograde cholangiopancreatography (ERCP), interventions such as: endoprosthesis (biliary stent) placement with or without concurrent sphincterotomy, balloon dilatation of anastomotic strictures, can be included[12].

Bile duct stones, bile leaks and anastomotic strictures are among the most common post-transplant complications reported[13-19]. The reported incidence of such complications among different centers has been variable[8,12,17]. Our institution has previously reported the endoscopic experience with BTC in the post-OLT patient, however data did not include management of a "redundant bile duct" (RBD) (Figure 1)[8].

We define the "RBD" a surgically reconstructed donor-recipient extrahepatic bile duct, which due to its length (longer than the native recipient duct), in the absence of anastomotic stricture, creates a looped, sigmoid-shaped ("S", "Z") appearance, which leads to delayed bile flow into the duodenum, functionally translating into cholestasis and abnormal pattern of the liver associated tests.

The term was described as an analogy to the "redundant colon", which describes a large intestine (colon) that is longer than normal and as a result has repetitive, overlapping loops. Typically, the "redundant colon" is a normal anatomic variation.

From our large transplanted data we present our endoscopic experience with the RBD treatment in the post-OLT patient. To our best knowledge, this is the first presentation of successful endoscopic management of the RBD in the post-OLT patient.

MATERIALS AND METHODS

We performed a retrospective analysis of records from the Transplant Clinic, Endoscopy and radiology of patients who underwent OLT at the Liver Transplant Center, University Health Science Center at San Antonio.

One thousand two hundred and eighty-two patient records who received OLT from 1992 through 2011 were reviewed. Patients with biliary T-tube placement, biliary anastomotic strictures, bile leaks, bile-duct stones-sludge and suspected sphincter of oddi dysfunction were excluded.

Patients who underwent ERCP in the post-transplant period, indication and number of procedures per patient were reviewed. Laboratory data and pertinent radiographic imaging noted included the pre-ERCP period and a follow-up period of 6-12 mo after the last ERCP intervention.

RBD was identified as a sigmoid-shaped bile duct on cholangiogram (Figure 1) with associated cholestatic liver biomarkers. Endoscopic intervention included biliary stent placement with or without sphincterotomy. All ERCP were performed by experienced biliary endoscopists.

The incidence of RBD, the number of ERCP corrective sessions, and the type of endoscopic interventions were recorded. Successful response to endoscopic therapy (resolution of RBD) was defined as normalization of cholestatic liver profile up to one year after last endoscopic intervention and resolution of cholangiographic abnormalities (Figure 2).

Table 1 Patient data demographics

Men	12
Women	9
Average age (yr)	59.6
Indication for OLT	
Hepatitis C	15
Cryptogenic	2
Steatohepatitis	1
Medication induced failure	1
Alcoholic cirrhosis	1
Autoimmune hepatitis	1
Average time (d) from OLT to ERCP	88.1
Indication for ERCP	
Cholestatic LFT	21/21

LFT: liver function test; OLT: Orthotopic liver transplantation; ERCP: Endoscopic retrograde cholangiopancreatography.

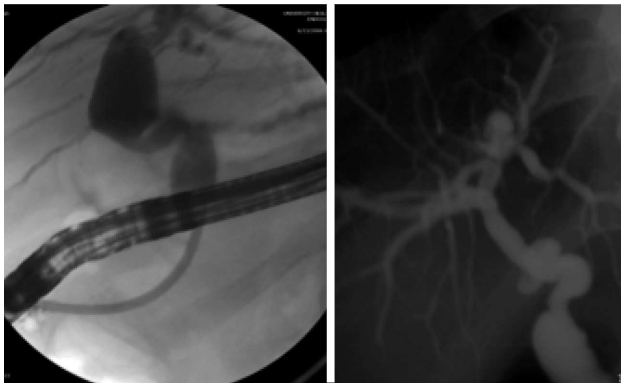

Figure 1 Cholangiogram of a redundant common bile duct.

Statistical analysis

Statistical analyses were performed with the SAS statistical software (version 9.2, SAS Institute Inc. Cary, NC). We used the χ^2 test to test whether categorical variables differed between individuals whose RBD resolved with ERCP and counterparts that failed ERCP intervention. Comparisons between the 2 groups for continuous variables were performed by using the Mann-Whitney *U* test (a nonparametric test). Results are reported as median and range or percentage as appropriate. Significance was assumed for $P < 0.05$ (2 sided).

RESULTS

Two hundred and twenty-four patients underwent ERCP for suspected BTC. RBD was reported in each of the initial cholangiograms by three individual experienced endoscopist (Patel S, Gross G, Rosenkranz L) and reviewed by the authors of the manuscript. Twenty-one out of 1282 (1.6%) of liver transplanted patients were identified as having RBD. Patient demographics are listed in Table 1. There were 12 men and 9 women, average age of 59.6 years. Primary indication for liver transplantation was end stage liver disease secondary to hepatitis C (71.4%). Primary indication for ERCP was cholestatic

Table 2 Interventions and results in 21 patients with redundant bile duct

Results	Resolved (n = 15)	Failure (n = 6)	P value
Men n (%)	8/15 (53.3)	4/6 (66.7)	0.577
Age[1], yr	59.0 (39.0-70.0)	64.5 (50-75)	0.094
Hepatitis C indication n (%)	11/15 (73.3)	4/6 (66.7)	0.760
Time from OLT to ERCP[1], d	14 (4-1059)	225 (8-865)	0.086
Total ERCP	3 (2-10)	3 (1-4)	0.492
Total biliary stents placed			
Average stent per patient[1]	3 (0-15)	2 (0-4)	0.475
Average stent per session[1]	1.0 (0-1.5)	0.9 (0-1)	0.602
ERCP sessions for resolution			-
Single session	6/15	-	
Two sessions	4/15	-	
> Two sessions	5/15	-	
Percutaneous biliary drainage		2	-
Choldocojejunostomy		2	-
T bili[1], mg/dL	5.0 (0.3-37.3)	6.1 (1.2-34.9)	0.586
AST[1]	122 (34-444)	190 (40-1131)	0.392
ALT[1]	248 (42-668)	262 (58-1579)	0.846
Alk phos[1]	460 (109-1066)	345 (243-936)	0.907

[1]Median (range). OLT: Orthotopic liver transplantation; ERCP: Endoscopic retrograde cholangiopancreatography; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

pattern of liver associated biochemical markers. Nineteen out of 21 patients underwent endoscopic therapy and 2/21 required immediate surgical intervention, for failure to stenting the bile duct. In the endoscopically managed group: 65 ERCP procedures were performed with an average of 3.4 per patient and 1.1 stent per session. Fifteen out of 19 (78.9%) patients were successfully managed with biliary stenting. Interventions and results are listed in Table 2. All stents were plastic. Selection of stent size and length were based upon endoscopist preference. Stent size ranged from 7 to 11.5 Fr (average stent size 10 Fr); Stent length ranged from 6 to 15 cm (average length 9 cm). Each stent remained in place for an average of 93 d. Concurrent biliary sphincterotomy was performed in 10/19 patients. Single ERCP session was sufficient in 6/15 (40.0%) patients, whereas 4/15 (26.7%) patients needed two ERCP sessions and 5/15 (33.3%) patients required more than two (average of 5.4 ERCP procedures). Single biliary stent was sufficient in 5 patients; the remaining patients required an average of 4.9 stents. Figure 3 represents a cholangiogram with multiple stents placed in a redundant bile duct. Four out of 19 (21.1%) patients failed endotherapy (lack of resolution of RBD and recurrent cholestasis in the absence of biliary stent) and required either choledocojejunostomy (2/4) or percutaneous biliary drainage (2/4). The medical records of the 15 successful endoscopically managed patients were reviewed for a period of one year after removal of all biliary stents. Eleven patients had continued resolution of cholestatic biomarkers (73%). One patient had recurrent hepatitis C, 2 patients suffered septic shock which was not associated with ERCP and 1 patient was transferred care to an outside provider and records

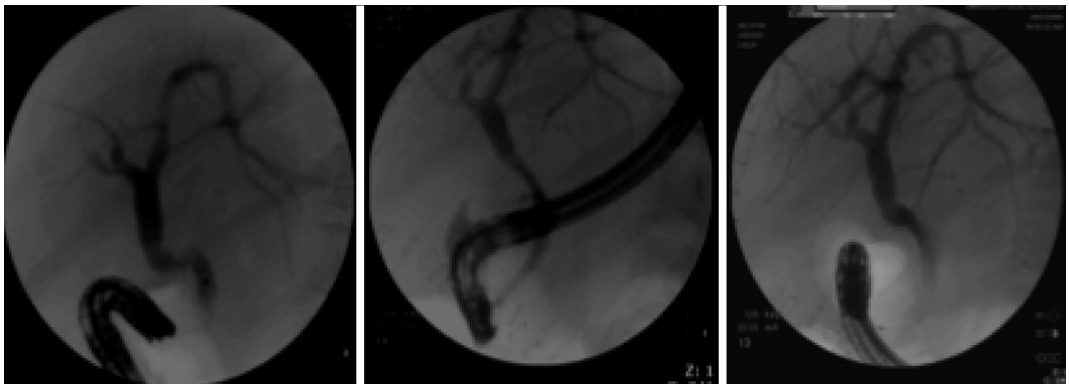

Figure 2 Single patient series of successful endoscopic management. Cholangiogram with redundant anastamosis. Placement of a 10 Fr by 9 cm plastic stent. Cholangiogram after stent removal with improvement in redundancy and normalization of cholestatic liver profile.

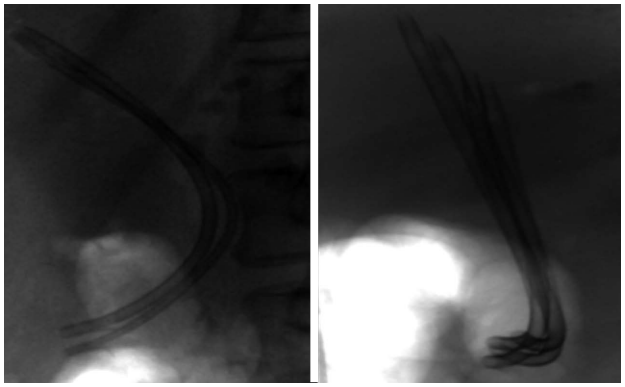

Figure 3 Multiple stent placement in a redundant bile duct.

were not available for our review. Endoscopic complications (ERCP-related) recorded included: 2/65 (3%) post-ERCP pancreatitis and 2/10 (20%) non-complicated post-sphincterotomy bleeding. No endoscopic related mortality was found.

DISCUSSION

Since their initial description, BTC remain a significant source of morbidity and mortality after OLT. Complication rates have been reported as s high as 20% in some series[18]. During organ procurement, the surgeon attempts to minimize any disruption of the donor bile duct blood supply using a variety of techniques[20-24]. During transplantion, surgeons approximate the donor liver and bile duct to the native bile duct stump with caution. A laparotomy pad is placed above the liver, in order to maintain proper positioning during anastomosis and once completed, the pad is removed and the liver allowed to retract cephalad into its natural position. The bile duct is anastomosed with a gentle tension in order to reduce the risk of ischemia and bile leaks. Additionally, torsion of the liver during the transplant may lead to tension and leaks. It should be known that the surgeons do not make special attempts to avoid redundancy. Clearly overt discrepancies are addressed, but this aspect of the operation is quick and concise.

These techniques are performed to preserve blood supply and may theoretically lead to less ischemic bile duct complications. The successful endoscopic management of biliary leaks, bile duct strictures and sphincter dysfunction has previously been reported however, to our best knowledge, this is the first report of successful endoscopic management of a RBD in the post-OLT patient. Although post-OLT RBD represents an uncommon complication with an incidence of 1.6%, endoscopic management appears to be a reasonable initial approach as 78.9% of patients with a RBD post-OLT can be successfully managed with a combination of biliary stenting and sphincterotomy. Endoprosthesis selection is based on the endoscopist preference and comprises plastic biliary stents of variable width (7-11.5 Fr) and length, therefore it is difficult to comment in a non-randomized retrospective study if stent size or length impacted the overall outcome. The exact mechanism of resolution remains unclear, however, we suspect that stent placement alters the configuration of duct anatomy thereby leading to a resolution of the redundant duct. Hepatobiliary biopsies pre and post stent placement would aid in the further evaluation of the histochemical changes associated with this entity[25,26]. However this was not the main endpoint but does represent an avenue of further research. One year follow up of bilirubin and liver associated enzymes also suggest that endoscopic treatment is a viable option as 73% had continued resolution of cholestatic of liver profile.

COMMENTS

Background

Since their initial description, biliary tract complications (BTC) remain a significant source of morbidity and mortality after orthotopic liver transplantation (OLT). Despite improvement in surgical techniques, the biliary reconstruction remains a sensitive area regarding graft and recipient complications. Endoscopic therapies have been effective in the management of BTC. Authors present their experience with "redundant bile duct" (RBD) in the post-OLT setting.

Research frontiers

Management of BTC in the post-OLT setting has previously been reported; however, endotherapy and outcomes in the management of the RBD has not

been described until present. The surgical management of the RBD has been published. Authors' group is the first to propose endoscopic management *via* a combination of biliary stenting and sphincterotomy as an initial approach to the RBD.

Innovations and breakthroughs

This is the first to demonstrate that a RBD can be successfully managed with a combination of biliary stenting and sphincterotomy with a 78.9% success rate at our institution. One year follow up data also suggests that endoscopic management confers a sustained response.

Applications

Although post-OLT RBD an uncommon complication, endoscopic management appears to be a reasonable initial approach.

Terminology

BTC include: leaks, strictures, retained stones and sphincter of oddi dysfunction. RBD is a surgically reconstructed donor-recipient extrahepatic bile duct which creates a looped, sigmoid-shaped ("S", "Z") appearance thereby resulting in delayed bile flow into the duodenum, OLT, Endoscopic Retrograde Cholangiopancreatography.

Peer review

This manuscript reports on an unusual problem which they have termed the RBD. They reference their own prior study which suggests that such an entity may not be widely known or even accepted. Given that this could represent a real entity, publication may be appropriate.

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BRIEF ARTICLE

Human leukocyte antigen *DQ2/8* prevalence in non-celiac patients with gastrointestinal diseases

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Abstract

AIM: To investigate the prevalence of human leukocyte antigen (HLA) *DQ2/8* alleles in Southern Italians with liver and gastrointestinal (GI) diseases outside of celiac disease.

METHODS: HLA *DQ2/8* status was assessed in 443 patients from three ambulatory gastroenterology clinics in Southern Italy (University of Federico Ⅱ, Naples, Loreto Crispi Hospital, Ruggi D'Aragona Hospital, Salerno). Patients were grouped based on disease status [pre-post transplant liver disease, esophageal/gastric organic and functional diseases, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)] and *DQ2/8* alleles, which correspond to a celiac disease genetic risk gradient. Subject allele frequencies were compared to healthy Italian controls.

RESULTS: One hundred and ninety-six out of four hundred and forty-three (44.2%) subjects, median age 56 years and 42.6% female, were *DQ2/8* positive. When stratifying by disease we found that 86/188 (45.7%) patients with liver disease were HLA *DQ2/8* positive, 39/73 (53.4%) with functional upper GI diseases and 19/41 (46.3%) with organic upper GI diseases were positive. Furthermore, 38/105 (36.2%) patients with IBS and 14/36 (38.9%) with IBD were HLA *DQ2/8* positive (P = 0.21). Compared to healthy controls those with functional upper GI diseases disorders had a 1.8 times higher odds of *DQ2/8* positivity. Those with liver disease had 1.3 times the odds, albeit not statistically significant, of *DQ2/8* positivity. Both those with IBS and IBD had a lower odds of *DQ2/8* positivity compared to healthy controls.

CONCLUSION: The proportion of individuals HLA *DQ2/8* positive is higher in those with liver/upper functional GI disease and lower in IBS/IBD as compared to general population estimates.

Key words: Human leukocyte antigen *DQ2/8*; Gastrointestinal and liver disease; Celiac disease

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INTRODUCTION

The human leukocyte antigen (HLA) class Ⅱ genes comprise a highly polymorphic region in the short arm of chromosome 6 and are responsible for the creation of molecules involved in exogenous antigen presentation to T cells[1,2]. A subset of class Ⅱ genes, encoding the *DQ2* and *DQ8* serotypes, have been frequently implicated in autoimmune disease pathogenesis. Prevalent in 30%-40% of healthy individuals, *DQ2* and *DQ8* are associated with diseases such as insulin-dependent diabetes mellitus and Hashimoto's Thyroiditis[3,4]. These haplotypes may be best characterized through the gluten dependent relationship with celiac disease, an autoimmune mediated enteropathy affecting approximately 1% of Europeans and North Americans[5-7]. Consequently, many studies have attempted to estimate or infer the proportion of celiac disease risk due to particular *DQ2/8* isoforms. For this reason, a genetic risk gradient has been recently characterized for *DQ2/8* allele variants[8]. The risk associated with celiac disease compared to those healthy depends, incrementally, on the number/type of HLA alleles possessed by an individual. Those with one or both of the *DQ2/8* alleles have a risk ranging from 1:7-1:35, while those lacking all potential immunogenic loci have a near zero chance of contracting celiac disease[8,9]. Beyond celiac disease risk, disease severity and anti-tTg antibody levels are thought to be further tied to this disease/gene-dose relationship[10].

There are several reasons why it may be prudent to study *DQ2/8* alleles in liver/gastrointestinal (GI) disease outside of celiac disease. First, evidence suggests that celiac disease may modify the risk of developing irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), eosinophilic esophagitis, or certain liver diseases[11-14]. Recent research has also shown the presence of HLA *DQ2/8* alleles by themselves, outside of celiac disease, to be associated with GI disease[15-17]. This suggests that *DQ2/8* haplotypes may act as a common factor in liver/GI disease pathogenesis; possibly through a similar mechanism to that of celiac disease. Furthermore, as *DQ2/8* haplotypes contain myriad genes involved in inflammatory processes, such as tumor necrosis factor-α, causal mechanisms between these genes and GI disease may exist[18]. Comparisons of *DQ2/8* prevalence in non-celiac GI diseases, however have not been directly studied.

DQ2/8 associated disease risk is known to be modified across individuals or populations varying in ethnic background, geography or gender[19-23]. Moreover, *DQ2/8* prevalence in Southern Italians has not been characterized. Thus, in this study we sought to first define the prevalence of *HLA DQ2/8* alleles in a Southern Italy non-celiac GI tertiary ambulatory clinic population. Subsequently, we desired to determine what HLA *DQ2/8* haplotypes, if any, were associated with specific liver/GI diseases.

MATERIALS AND METHODS

Subject population

Patients (n = 463) from the gastroenterology ambulatory clinics of three hospitals were recruited over a period of three months. Three hundred and twenty-two subjects were recruited from University of Federico Ⅱ, Naples, Italy, 85 from Loreto Crispi Hospital, Naples, Italy and 56 from Ruggi D'Aragona Hospital, Salerno, Italy. During consultation patient's demographics and disease history were recorded. Disease status was classified according to the nature of presenting problem. Separate categories were attributed generally to pre- or post-liver transplant treatment for chronic viral hepatitis, upper functional and organic GI (gastritis, esophagitis) diseases, lower functional (IBS) and lower inflammatory GI (IBD) diseases. Those with IBS were diagnosed *via* Rome Ⅲ criteria. Overall population characteristics are described in Table 1. Participants were excluded from this study if they had missing data on disease status, multiple upper GI diseases or a prior diagnosis of celiac disease (n = 20).

Informed consent was obtained from each patient or patient guardian prior to study enrollment. The study was approved by ethics review board of the University of Naples "Federico Ⅱ" and complied with the Helsinki Ⅱ declaration.

Sample collection and analysis

Peripheral blood was collected in ethylene-diamino-tetra-acetic tubes and stored at 4 ℃. Genomic DNA was isolated and polymerase chain reaction with sequence-specific primer was then performed to test solely for the presence/absence of *DQ2/8* genes (Celiac Gene Screen, BioDiagene, Palermo, Italy). If patients were "susceptible to celiac disease", further analysis was performed to discern specific alleles known to be associated with celiac disease risk (Celiac Gene Alleles, BioDiagene, Palermo, Italy). Fluorescence detection of *DQ2/8* was performed using BioRun Reader (Celiac Gene Alleles, BioDiagene, Palermo, Italy). Patients "susceptible to celiac disease" are generally understood to have at least one of the HLA *DQ2/8* alpha or beta alleles.

Using fluoro-immuno-assay, with human recombinant tTg as an antigen, patients positive for *HLA DQ2/DQ8* alleles were tested for anti-tTg antibodies (a-tTg) and adequate immunoglobulin A (IgA) levels (CeliKey IgA, EliA, Phadia Freiburg, Germany). Anti-tissue transglutaminase levels greater than 10 (EliA U/mL) were considered positive. Those values between 7 and 10 (EliA U/mL) were considered equivocal and those less than 7 (EliA U/mL) negative. In later analysis both positive and equivocal groups were combined to increase power. None of the patients tested for total IgA were found to be deficient. Due to laboratory error several (n = 46) patients' a-tTg levels were unattainable.

Healthy controls

In order to compare the distribution of *DQ2/8* alleles

Table 1 General demographic attributes of study population

	Overall	Liver	Upper functional	Upper organic	IBS	IBD
Total number	443	188	73	41	105	36
Gender						
Male	44.9%	62.8%	27.4%	34.2%	27.6%	50%
Female	55.1%	37.2%	72.6%	65.8%	72.4%	50%
Age (yr)						
Median	56	61	50	57	43	32
Range	72	66	71	66	62	60
Quartiles						
14-41	-	4.81%	30.1%	24.4%	45.6%	55.6%
42-56	-	23.5%	28.8%	24.4%	28.2%	19.4%
57-65	-	38.5%	21.9%	12.2%	10.7%	8.3%
66-86	-	33.2%	19.2%	39%	15.5%	16.7%

IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

in study participants to the general Italian population we incorporated data from a prior published study by Megiorni *et al*[21]. Healthy participants consisted of 292 healthy and 259 family based controls from Rome, Italy. The prevalence of *DQ2.5/8* in healthy controls was 29%. This increased to 39% after incorporating the less common *DQ2* isoforms.

HLA classification

DQ2 and *DQ8* serotypes, if indicated, were tested for the following alleles: *DQA1*0201*, *DQA1*03*, *DQA1*05*, *DQB1*02*, *DQB1*0301/0304* and *DQB1*0302/0305*. The following *DR* alleles were typed in order to determine the presence of *DQ/DR* haplotypes: *DRB1*03*, *DRB1*04*, *DRB1*07*, *DRB1*11*, *DRB1*12*.

DQ2/8 haplotypes were classified by Megiorni *et al*[8]. *DQ2* positivity was defined as *DQA1*05* in cohort with *DQB2*02* (*DQ2.5*), or *DQA1*0201* (*DQ2.2*)/*DQA1*03* (*DQ2.3*) with *B1*02*. *DQ8* positivity was defined as *DQA1*03* with *DQB1*0302*.

Statistical analysis

The Pearson χ^2 test was performed on categorical data regarding demographics and the overall relationship of prevalence data. Fisher's exact test was used for analysis of data with cell counts $n < 5$. Basic tabular analysis was also performed to obtain odds ratios. A cut-off of $P = 0.05$ was considered significant; all intervals were reported at 95% confidence. The analysis was performed using SAS 9.2 and SPSS 19.

RESULTS

We performed a cross-sectional analysis of HLA *DQ2/8* allele prevalence in a Southern Italian population of patients afflicted with either liver or other digestive diseases outside of celiac disease. *DQ2/8* haplotypes were stratified using a prior defined risk gradient relevant to celiac disease and prevalence in our disease population was compared to estimates in healthy controls.

Table 2 Prevalence of human leukocyte antigen *DQ2/8* by age, gender and gastrointestinal disease

	Proportion of positive subjects	Prevalence	*P* value
Overall	196/443	44.2%	0.02
Gender[1]			
Male	92/199	46.2%	
Female	104/244	42.6%	0.45
Age (yr)			
14-41	48/108	44.4%	
42-56	42/111	37.8%	
57-65	53/107	49.5%	
66-85	52/114	45.6%	0.37
Disease groups			
Liver	86/188	45.7%	
Upper functional	39/73	53.4%	
Upper organic	19/41	46.3%	
IBS	38/105	36.2%	
IBD	14/36	38.9%	0.21

[1]$P > 0.05$ for differences between genders in each disease group except for those with upper functional disorders. In these patients significantly more males were positive than females ($P = 0.05$). IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

Prevalence of HLA alleles in study population

From the patients included in our analysis, 196/443 (44.2%; 95%CI: 39.6%-48.9%) were considered to be HLA *DQ2/8* positive, regardless of disease status. Within those who were positive 144/197 (73.1%) had *DQ2.5* and/or *DQ8*. Table 2 details the prevalence of HLA *DQ2/8* by age, gender and GI disease in the study's participants. The overall difference in *DQ2/8* prevalence between these disease groups was not statistically significant ($P = 0.21$).

Comparison of DQ2/8 prevalence in study population to healthy controls

Subjects *DQ2/8* alleles were organized highest to lowest genetic risk of celiac disease, as described in Megiorni *et al*[8] and compared to healthy controls. No statistically significant difference in HLA *DQ2/8* prevalence between our subject population and healthy controls was found ($P = 0.16$). As with healthy controls, study subjects clustered towards lower celiac disease risk *DQ2/8* alleles with *DQ2.5* heterozygotes lying in the majority. Odds ratio calculations revealed that those with functional gastric/esophageal disorders had a 1.8 fold higher odds of being HLA *DQ2/8* positive as compared to healthy controls. Patients with organic gastric/esophageal disorders had 1.3 higher odds of *DQ2/8* positivity as compared to healthy controls. The odds were also increased in the liver disease group and decreased in IBS/IBD groups although these values were not significant (Table 3).

α-tTg

Out of the patients with a-tTg data available no a-tTg positive patients were found in the liver disease/transplant and inflammatory bowel group. One out of sixty-

Table 3 Magnitude of associations between human leukocyte antigen positivity and specific gastrointestinal disease

Disease group	Odds ratio	95%CI
Overall	1.2	0.96-1.6
Liver	1.3	0.94-1.8
Upper functional	1.8	1.1-2.9
Upper organic	1.3	0.71-2.7
IBS	0.89	0.57-1.4
IBD	0.99	0.49-1.9

IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

Table 4 Positive anti-tTg subject stratified by disease group

Disease group	*n*	Prevalence
Liver	0/173	0%
Upper functional	1/64	1.54%
Upper organic	0/35	0%
IBS	4/90	4.26%
IBD	0/36	0%

$P = 0.04$, for any difference in prevalence of anti-tTg positive between disease groupings. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

four (1.54%) subjects with functional gastric/esophageal issues and 4/90 (4.26%) with lower functional syndrome were found to be positive ($P = 0.04$; Table 4).

DISCUSSION

The clinical importance of HLA genetic testing has been established in several diseases[3,24]. In this study we performed a cross-sectional analysis on a Southern Italian population with the goal of investigating the prevalence of several HLA *DQ2/8* serotypes in those with GI issues outside of celiac disease.

HLA *DQ2/8* prevalence in Italy is thought to be between 30% and 40%, although this estimate may vary by geographic subpopulation[8,24]. Nearly half of the subjects in this study (44%) were considered HLA *DQ2/8* positive. A lesser proportion of those with IBS/IBD were HLA *DQ2/8* positive although these differences were not significant ($P = 0.21$) (Table 2). Within those who were HLA *DQ2/8* positive the majority possessed low risk celiac disease alleles (Table 5). Thus, our results suggest that *DQ2/8* haplotypes may play a role in liver/digestive disease through pathological mechanisms different from those of celiac disease.

Several studies have established significant associations between *DQ2*, primary sclerosing cholangitis and hepatitis C virus recurrence after transplant[25,26]. The large proportion (46%) of *DQ2/8* positive viral hepatitis patients in our study population agrees with the hypothesis that these haplotypes may be involved in certain liver disease pathogenesis.

Differentiating between functional and organic GI disease can be difficult yet is important due to the impacts on clinical decision-making. In this study we stratified our patient population based on upper and lower functional or organic GI disorders. Several studies have directly compared *DQ2/8* haplotype prevalence in upper organic GI disease. Lucendo *et al*[14] previously demonstrated a null association between *DQ2/8* and eosinophilic esophagitis. Interestingly, *DR3* and *DR4* have been significantly linked with atrophic gastritis in a similar Italian population[27]. The positive yet non-significant relationship between our organic gastric/esophageal patients and *DQ2/8* may be a consequence of the various upper GI organic diseases captured in our patient population. Unfortunately, due to sample size restrictions, we were not able to stratify by specific disease. Ultimately these findings suggest that it may be inappropriate to generalize upper organic GI disease as one confluent group because it is unknown whether the lack of a significant association was truly due to causal or confounding disease factors.

Table 5 Prevalence of specific human leukocyte antigen *DQ2/8* alleles between gastrointestinal and control populations *n* (%)

Overall	Gastrointestinal	Controls	Risk
DQ2 and *DQ8*	2 (0.45)	1 (0.2)	1:45
DQ2, *β1*02/*02*	17 (3.8)	13 (2.4)	1:63
DQ8, *β1*02* positive	8 (1.8)	4 (0.7)	1:39
β2, *β1*02/*02*	11 (2.5)	2 (0.4)	1:16
DQ2, *β1*02*/X	85 (19.2)	106 (19.2)	1:100
DQ8, *β1*02* negative	32 (7.2)	36 (6.5)	1:90
β2, *β1*02*/X	41 (9.3)	53 (9.7)	1:104
α5 + other	247 (55.8)	336 (60.9)	1:101
Total	443	551	

Omnibus *DQ2/8* positive *vs* *DQ2/8* negative χ^2; $P = 0.16$. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

Functional GI disorders represent the majority of GI cases yet many have etiologies, which are poorly understood. Whether it is genetic abnormalities, psychological factors or other environmental variables, functional disorders can represent complex, difficult to solve cases[28]. The strongest evidence of an association with *DQ2/8* in this study was found in patients with functional upper GI disorders. Patients in this study had 1.8 higher odds of *DQ2/8* positivity if they had an upper functional GI disorder as compared to healthy controls. This may signify that the risk of functional upper GI onset or recurrence is modified by the presence of particular *DQ2/8* haplotypes. Currently, the only published data, which could be used to comment on these findings, relates to celiac disease and *DQ2/8*. For example, Ford *et al*[29] conducted a meta-analysis, which found no association between celiac disease and functional dyspepsia. Overall, it is too early to decide whether *DQ2/8* could be used to differentiate functional *vs* organic disease or at least be incorporated into a clinical algorithm that dictates likelihood of disease.

Known immunological associations between IBD and *DR7*, which is linked to both *DQ2* and *DQ8* hap-

lotypes have been established[17,30]. Prior prevalence data though suggests that IBD, particularly Crohn's disease, is lower in individuals with the *DQ2/8* linked celiac disease[12]. The relative modest prevalence of IBD (39%) in our study supports this notion. IBS has also been linked to HLA *DQ2/8* haplotypes and bowel transit speeds[16,31]. Additionally several studies have demonstrated that those with IBS and *DQ2/8* positivity tend to present with symptoms indicative of gluten sensitivity and are responsive to a gluten-free diet[31,32]. Out of all of the disease groupings those with IBS had the lowest prevalence of *DQ2/8* positivity (36%). As those on a gluten-free diet were excluded from this study this may account for the low prevalence *DQ2/8* positivity in those with IBS.

A small part of this study wished to obtain a baseline level of a-tTg positive patients in a previously diagnosed GI population (Table 4). If these patients were assumed to have celiac disease, these results correspond with known prevalence estimates of the disease[5].

Those who had prior diagnosed celiac disease or were adhering to a gluten-free diet were excluded from the study. It is thought to be common for patients with general abdominal pain, diarrhea and/or nausea to experiment with a gluten-free diet in an attempt to ameliorate symptoms[33]. As such, removing individuals who may have experimented with this type of diet eliminated a potentially large source of bias. An additional strength of this study was the precision with which HLA haplotypes and disease types were measured. Barring laboratory error, the HLA typing assays in this study have been shown to have near perfect sensitivity and specificity[34]. During blood collection patient's disease status was recorded. Thus, it was also unlikely that the classification of disease was subject to recall bias.

This study aimed to generally define HLA *DQ2/8* prevalence in diseases/disorders that may be linked to celiac disease. As such there were several limitations, which could have potentially biased the results. The cross-sectional nature limited the collection of subject's lifestyle and disease history. Therefore, data such as age of disease onset and severity were unavailable. The Southern Italian population is typically generically and environmentally homogenous thus unmeasured confounders would not significantly influence the results. Controls from the study were described as "healthy". We know from Megiorni *et al*[8] that these participants did not have celiac disease but it is possible they were afflicted with a liver or GI disease. This type of bias would have most likely pushed the magnitude of our estimates towards the null, masking potential associations. The small sample size in our study also limited our ability to make statistically significant conclusions and investigate specific *DQ2/8* allele associations.

Due to the limitations of the present study it may be difficult to make truly suggestive conclusions regarding the relationship between HLA *DQ2/8* positive patients and liver/GI conditions. This study though has taken the first step through implying a potential association between specific HLA *DQ2/8* alleles and GI disease pathogenesis. Reproducibility of these results may eventually lead to the creation of clinical markers of elusive disease onset, such as in IBS or other clinically ambiguous disorders[11,16,28,35,36]. Future studies should involve expanding the number of study participants in order to look at specific *DQ2* alleles or investigating the shared influence of non-HLA celiac disease risk alleles.

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COMMENTS

Background

Human leukocyte antigen (HLA) *DQ2/8* alleles and associated haplotypes are important players in the pathogenesis of several autoimmune diseases, particularly celiac disease. The distribution of *DQ2/8* alleles in gastrointestinal (GI) disease outside of celiac disease though has been poorly established.

Research frontiers

HLA *DQ2/8* alleles in patients can be easily tested with currently laboratory capabilities. Knowledge of HLA *DQ2/8* frequencies related to autoimmune disease is being used to identify high risk populations and drive clinical recommendations regarding *DQ2/8* gene testing.

Innovations and breakthroughs

Similar studies have described HLA *DQ2/8* risk alleles in diseases such as type 1 diabetes or celiac disease. For example, in celiac disease those with one or more of the *DQ2.5/8* alleles are at highest risk of disease onset. To date no studies have directly compared *DQ2/8* prevalence in GI disease outside of celiac disease. By comparing the prevalence of HLA *DQ2/8* to that of healthy controls we demonstrated that, like celiac disease, those with liver disease and esophageal/gastric disorders (both organic and functional) are more likely to be *DQ2/8* positive.

Applications

Although this study is preliminary in nature, the results suggest that the development of novel clinical susceptibility markers of GI disease may exist. Particular *DQ2/8* polymorphisms may point to increased risk of certain liver or esophageal/gastric disease.

Peer review

The study investigated the prevalence of HLA *DQ2/8* alleles in Southern Italians with liver and GI diseases outside of celiac disease. The proportion of individuals HLA *DQ2/8* positive resulted higher in those with liver/gastric or esophageal GI disease and lower in irritable bowel syndrome/inflammatory bowel disease as compared to general population estimates. The study is well designed and conduced.

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BRIEF ARTICLE

Gastroesophageal reflux disease after diagnostic endoscopy in the clinical setting

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Abstract

AIM: To investigate the outcome of patients with symptoms of gastroesophageal reflux disease (GERD) referred for endoscopy at 2 and 6 mo post endoscopy.

METHODS: Consecutive patients referred for upper endoscopy for assessment of GERD symptoms at two large metropolitan hospitals were invited to participate in a 6-mo non-interventional (observational) study. The two institutions are situated in geographically and socially disparate areas. Data collection was by self-completion of questionnaires including the patient assessment of upper gastrointestinal disorders symptoms severity and from hospital records. Endoscopic finding using the Los-Angeles classification, symptom severity and it's clinically relevant improvement as change of at least 25%, therapy and socio-demographic factors were assessed.

RESULTS: Baseline data were available for 266 patients and 2-mo and 6-mo follow-up data for 128 and 108 patients respectively. At baseline, 128 patients had erosive and 138 non-erosive reflux disease. Allmost all patient had proton pump inhibitor (PPI) therapy in the past. Overall, patients with non-erosive GERD at the index endoscopy had significantly more severe symptoms as compared to patients with erosive or even complicated GERD while there was no difference with regard to medication. After 2 and 6 mo there was a small, but statistically significant improvement in symptom severity (7.02 ± 5.5 *vs* 5.9 ± 5.4 and 5.5 ± 5.4 respectively); however, the majority of patients continued to have symptoms (*i.e.*, after 6 mo 81% with GERD symptoms). Advantaged socioeconomic status as well as being unemployed was associated with greater improvement.

CONCLUSION: The majority of GORD patients receive PPI therapy before being referred for endoscopy even though many have symptoms that do not sufficiently respond to PPI therapy.

Key words: Gastroesophageal reflux disease; Epidemiology; Proton pump inhibitor; Acid suppressive therapy; Endoscopy; Barrett's esophagus; Functional gastrointestinal disorders

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INTRODUCTION

Heartburn and/or acid regurgitation occurring at least weekly, is very common in the general population[1]. Gastroesophageal reflux disease (GERD) is defined as typical symptoms occurring 2 or more times weekly, or symptoms perceived as problematic to patients, or resulting in complications[2,3].

Many clinical trials have shown that proton pump inhibitors (PPIs) are highly effective for healing of erosive GERD, and controlling symptoms[4]. Reflux symptoms are common, and treatment is readily available and regarded as highly efficacious. Thus, consistent with current guidelines many sufferers have therapy first, and are only referred for investigation (*i.e.*, endoscopy) if treatment fails or symptoms relapse. PPI are currently the most effective therapy for GERD, although cost effectiveness[5], risks in long term treatment[6,7] and their role in endoscopy-negative reflux disease are open to discussion[8].

In the highly controlled clinical trial environment patients who do not respond to PPI therapy are typically excluded. Thus clinical trials may not mirror routine clinical care when patients are referred for endoscopy because symptoms are not controlled and clinicians might be left with an unrealistic expectation of treatment efficacy. Moreover, in routine clinical care settings, there are a number of confounders that may interfere with, or modulate, the response to therapy for reflux symptoms. While changes in lifestyle habits such as weight loss, smoking cessation and reduction of alcohol consumption are often advised[9] very little is known about the role of body mass index, alcohol and smoking or socioeconomic status (SES)[10] on the response to therapy in real life. Whilst lifestyle factors have been related to the risk of having reflux[1], it is unclear whether they affect the response to therapy. There are now sufficient data to show that less that 50% of patients with typical GERD symptoms have mucosal lesions. The remainder are referred to as patients with non erosive gastroesophageal reflux disease (NERD). While some of these patients may have an increased acid exposure without mucosal lesions, other patients may not have an increase esophageal acid exposure and moreover, their heartburn symptoms might not be associated with episodes of esophageal acid exposure[11].

We therefore sought to determine and quantitate in a routine clinical setting in patients with GERD symptoms referred for endoscopy: (1) the symptom intensity and the improvement of symptoms to therapy (with PPIs); (2) the relation between symptoms and treatment response in relation to underlying structural lesions; and (3) whether lifestyle factors or socio-demographic variables affect this response in patients presenting for endoscopy because of reflux symptoms.

MATERIALS AND METHODS

Study design/overall approach

During a 24 mo period, patients referred, for endoscopic assessment of reflux symptoms at two large metropolitan hospitals were invited to participate in this observational study. The two institutions, the Royal Adelaide Hospital (RAH) and the Lyell McEwin Hospital (LMH) were both located in a single metropolitan Area Health Service (Central Northern Adelaide Health System, CNAHS), however they are located within geographically (approximately 25 km apart) and socially disparate areas[12]. The CNAHS serves a metropolitan and semi-rural population of more than 760000 residents. Both endoscopy units accept direct referrals from primary care doctors for upper gastrointestinal endoscopy (UGIE). All endoscopists were board certified with more extensive experience in diagnostic and therapeutic endoscopy. State of the art (Olympus) equipment was used.

This study did not include interventions other than obtaining informed consent und assessing symptoms and other parameters at baseline and during the follow-up. In particular there was no interference with normal care provided by general practitioners and specialists or interactions of the study staff that could shift attention towards symptoms or enhance compliance. Patients referred for UGIE with the primary complaints of typical reflux symptoms (heartburn +/- acid regurgitation) recorded as the indication on the referral for UGIE, between 18 and 65 years of age and capable of completing questionnaires were invited to participate. Exclusion criteria included significant medical co-morbidities (American Society of Anesthesiologists Ⅲ or Ⅳ or reduced life expectancy), pregnancy and unstable psychiatric disorder and inability to read and write or communicate in English.

The study was designed as a prospective, observational study with no interference with routine clinical management. Patients were invited and consented on the day of UGIE and completed the survey at baseline and two and six mo after the initial assessment. Data collection was by self-completion of questionnaires and from hospital records. In order to avoid any interference with the study objectives, patients were only contacted once at the defined follow-up time points, no measures to increase compliance with medication or behavioural interventions outside routine clinical care were provided.

The study was approved by the human research ethics committees of both hospitals, and each patient gave informed consent. The study was registered at the Australian New Zealand Clinical Trials Registry as "Multivariate analysis of predictors for severity of mucosal lesions in patients with gastro-oesophageal reflux symptoms: a clinical, epidemiological and endoscopic survey" (ACTRN12609000045213).

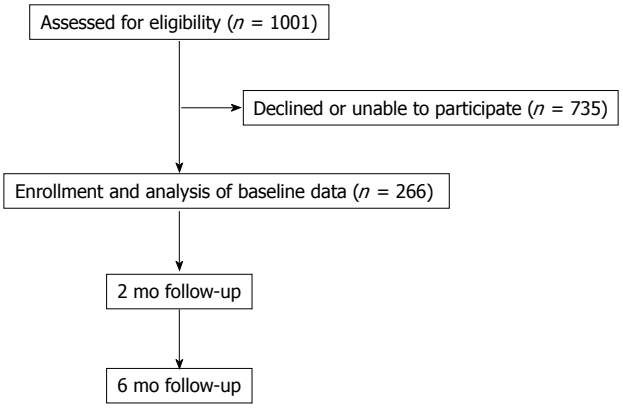

Figure 1 Consort diagram.

Treatment, lifestyle and sociodemographic data collection

In addition to the questionnaires (see below), hospital records and UGIE reports were reviewed to collect relevant data. Endoscopic findings at baseline were recorded using the Los Angeles (LA) classification[13]. The SES of the patients was established according to patients' residential postcodes using the Social Health Atlas of South Australia[12] and patients categorised into one of 3 groups: advantaged, average and disadvantaged.

Survey instruments

The postal survey included the patient assessment of upper gastrointestinal disorders symptom severity[14,15]. A symptom severity score (SSS) was calculated from it using 4 items covering reflux symptoms during the past week. The items used were: (1) heartburn (burning rising in your chest or throat) during the day; (2) regurgitation or reflux (fluid or liquid from your stomach coming up into your throat) during the day; (3) heartburn (burning rising in your chest or throat) at night (when recumbent); and (4) regurgitation or reflux (fluid or liquid from your stomach coming up into your throat) at night (when recumbent).

Severity of the symptoms was rated on a 6-point Likert scale, ranging from "none" = 0 to "very severe" = 5, therefore the SSS could range from 0-20. This score was used as the outcome variable to assess patients' reflux symptom severity over time. For this instrument we defined a clinically relevant improvement as change of at least 25% of symptom severity.

Statistical analysis

Means, standard deviations and percentages were calculated. *t*-tests or when appropriate non-parametric test were used to compare characteristics of patient groups. To assess which factors may affect symptom severity over time, bivariate correlations and non-parametric tests (Mann-Whitney) were used between individual factors and the change in SSS from baseline to 2 and 6 mo. Variables which were significant in initial analyses, or thought to be biologically relevant, were then included in a multivariable logistic regression model. Two-sided *P* values less than 0.05 were regarded as statistically significant. SPSS 15 was used for all analyses (2006, SPSS Inc., Chicago, IL, United States). A sample size of > 100 subjects was considered sufficient to identify relevant effect at an alpha level of 0.05 and a beta > 0.75.

RESULTS

Patient numbers and flow

Across the 2 sites, 1001 patients were eligible to participate (598 at the RAH; 403 at the LHM), (52.2% female overall). In total, 266 participated, 173 from the RAH [response rate (RR) = 29%] and 93 from LMH (RR = 23%) (P = 0.35), 145 participants were female (54.5%). Patient flow and follow-up are shown in Figure 1.

Baseline demographic details are shown in Table 1. Of note, at baseline 63% of participants were on treatment with a PPI (while all patients had PPI for at least 4 wk in the past), almost one third were regular smokers and 15.6% had high alcohol intake (daily or greater than 5 standard drinks/d).

As shown in Table 2 participants from the community hospital were significantly older, more socioeconomically disadvantaged, had more NERD, included fewer Barrett's surveillance cases, were more often smokers and had a trend for a greater proportion to be obese than those at the city hospital. However, there were no significant differences in gender mix or the proportion with high alcohol consumption between the hospitals.

Baseline endoscopic findings and symptoms

Higher grades of esophagitis (LA grade C + D) were associated with male gender (M = 38.8% *vs* F = 18.6%, P < 0.001), older age (P = 0.014) and with heavy alcohol consumption (15/37 with heavy alcohol consumption *vs* 15/125 with nil or moderate alcohol consumption, P = 0.002). Interestingly, there was an inverse association between higher grades of esophagitis and current smoking (15.4% in smokers *vs* 29.9% in non-smokers, P = 0.017).

The cohort had a mean SSS of 7 (SD 5.5) out of a possible maximum of 20. Reflux symptoms were rated as moderate to severe by 22.1% of the patients. In patients with NERD, the symptom score was significantly higher than in those with erosive or complicated GERD (*e.g.*, Barretts, Figure 2). Unemployed participants had a significantly higher mean symptom score at baseline than those who were employed (P = 0.007). Similarly, subjects with a lower SES had significantly more severe symptoms at baseline (8.1 ± 0.66) as compared to other patients (5.8 ± 0.52, P < 0.05); this difference was not explained by variation in the use of antisecretory drugs at baseline (P > 0.4) and overall there was no significant difference the in symptom score at baseline for patients with and without PPI therapy (without PPI 6.8 ± 0.75 *vs* with PPI 7.1 ± 0.47).

Follow-up

At 2 mo, 128 patients (48.1% of initial responders) agreed

Table 1 Baseline clinical and demographic data

LA-classification	NERD	Grade A	Grade B	Grade C	Grade D
n (F)	138 (90)	21 (13)	33 (15)	19 (5)	55 (22)
(%, 95%CI)	(51.9, 45.9-57.8)	(7.9, 5.2-11.8)	(12.4, 9.0-16.0)	(7.1, 4.6-10.9)	(20.7, 16.2-25.9)
Age, yr, mean ± SD (range)	49 ± 12.2 (19-65)	42 ± 12.9 (19-62)	50 ± 11.9 (22-65)	46 ± 12.8 (29-65)	53 ± 8.1 (37-65)
BMI, kg/m^2, mean ± SD (range)	28.8 ± -6.4 (19-49)	30 ± 8.5 (21-53)	29.7 ± 6.1 (21-44)	26.3 ± 5.5 (15-35)	28.7± 7.6 (16-53)
Percentage BMI > 30 kg/m^2	39.7%	38.9%	43.3%	27.8%	24.5%
PPI at enrolment	61.5%	50%	71%	57.9%	70.4%
Tobacco	25.2%	30%	8.7%	21.4%	7.9%
Alcohol (daily or > 50 g/d)	7.7%	13.3%	23.8%	14.3%	34.2%
Employed	54.4%	66.7%	55%	50%	44.7%
Married	48.6%	40%	52.2%	42.9%	51.3%
Socioeconomically disadvantaged	54.9%	56.3%	50%	43.8%	51.2%

LA: Los-Angeles; NERD: Non erosive gastroesophageal reflux disease; BMI: Body mass index; PPI: Proton pump inhibitor.

Table 2 Baseline comparisons of patients from the different study sites

Variable	RAH-tertiary referral centre (*n* = 173)	LMH-community Hospital (*n* = 93)	*P* value
Age, yr, mean ± SD (range)	48 ± 12.5 (19-65)	51.5 ± 10.2 (23-65)	0.027
Disadvantaged SES	56%	74.4%	< 0.001
LA-Grade NERD	46.8%	61.3%	0.024
LA-Grade C/D	21.4%	6.4%	0.024
Smoking	14.7%	31.3%	0.007
Alcohol (daily or > 5 Std. drinks/d)	17.1%	12.7%	0.701
Male gender	48.6%	60.2%	0.171
BMI > 30 kg/m^2	32.1%	44.1%	0.064

SES: Socioeconomic status; LA: Los-Angeles; NERD: Non erosive gastroesophageal reflux disease; BMI: Body mass index; Std.: Standard; RAH: Royal Adelaide Hospital; LMH: Lyell McEwin Hospital.

to be reassessed and 94 complete questionnaires were returned. At 6 mo, 108 participants (40.6% of initial responders) responded, providing 77 completed questionnaires.

Descriptive and univariate comparisons: During follow-up the vast majority of patients had PPI therapy. Only 9% never had PPI, and only 6% started PPI after the baseline visit. The overall mean symptom score significantly improved from baseline at two and six months (Figure 3). However, looking at individual improvements, only 15% of patients had improvement in their symptom score at 2 mo and 19% at 6 mo. The majority of subjects had residual reflux symptoms and 19% and 17% still rated their reflux symptoms as moderate to severe, at 2 and 6 mo respectively, compared to 22.1% at baseline. At 2 mo, a higher body mass index (BMI) correlated with a greater improvement in SSS (mean ± SD, 6.7 ± 28.8, P = 0.031). Only minor gender differences were noted; with a greater change of the absolute SSS from baseline to 2-mo seen in women compared to men (mean ± SD, 1.1 ± 4.3, P = 0.046), however there was no gender difference in symptom responsiveness at the 6-mo evaluation.

Multiple linear regressions: Multiple linear regression analyses were separately performed for the 2- and 6-mo time-points to identify factors were associated with changes in symptom severity over time. Factors included in the model were SES, BMI > 30 kg/m^2, PPI use, tobacco smoking, alcohol consumption, marital and employment status. Neither model (2 or 6 mo) reached overall significance (P = 0.104, adjusted R^2 = 0.066 at 2 mo; P = 0.732, adjusted R^2 = -0.043 at 6 mo); indicating that the chosen set of socio-demographic and life style factors did not explain a significant proportion of the variability in change in symptom severity.

Amongst individual predictors; social status and employment status were each associated with significant improvement in symptoms score at 2 mo. Patients of advantaged social status had an average 3.3 points greater improvement in symptoms score than patients of average SES and 2.5 points gain on those of disadvantaged SES (P = 0.031 and 0.014 respectively). Unemployed patients had an average 2.2 points greater SSS improvement compared to those employed or studying (P = 0.02). No individual factors were significantly associated with change in symptom score from baseline to 6 mo.

DISCUSSION

The main findings of this study are: (1) In the routine clinical setting more than 90% of patients referred for the assessment of suspected GERD are or have been on treatment with a PPI by the time of endoscopy. Nevertheless slightly more than 50% (138/266) do not have

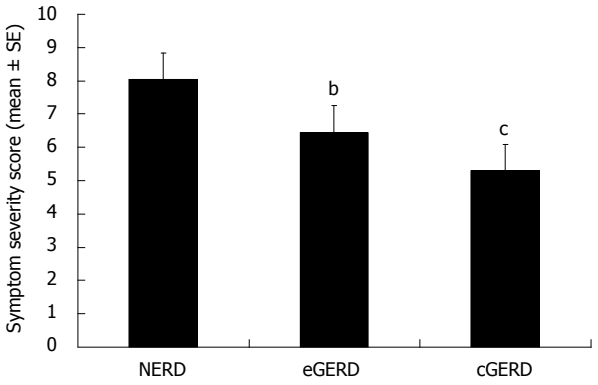

Figure 2 Mean symptom severity score for patients with non erosive reflux disease, erosive gastroesophageal reflux disease and complicated gastroesophageal reflux disease. Complicated gastroesophageal reflux disease (cGERD) includes all patients with Barretts esophagus. $^{b}P < 0.01$ *vs* non erosive reflux disease (NERD); $^{c}P < 0.05$ *vs* erosive gastroesophageal reflux disease (eGERD) and NERD.

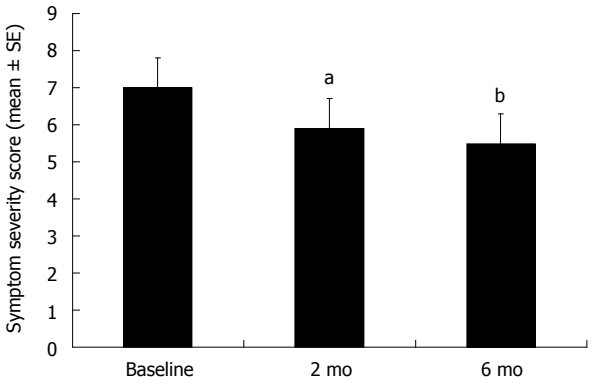

Figure 3 Mean symptom severity score at baseline and after 2 and 6 mo. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* baseline.

any mucosal lesions; (2) Patients without mucosal lesions have significantly more severe symptoms as compared to patients with erosive or complicated GERD; and (3) There is statistically significant improvement of symptoms over 6 mo. However, while the available treatments (*e.g.*, PPI) are considered highly effective for the healing of lesions and relief of symptoms, it is remarkable that the majority of patients continue to have GORD symptoms.

It is important to note that the majority of patients referred for endoscopic assessment of GERD symptoms, received PPI therapy at the time of endoscopy or had received PPI before. In spite of that 48% of patients were found to have mucosal lesions at the time of endoscopy. However, symptoms do not appear to be "driven by lesions" as symptom severity was significantly higher in patients without mucosal lesions as compared to those with lesions. Moreover, symptoms persisted in the majority of patients although there was a modest, even though statistically significant improvement of symptoms during follow-up.

Previous clinical trials have clearly shown that GERD patients can be effectively treated with PPI[16]. While there is no reason to question the data of these clinical trials, the typical patient now referred in the routine clinical setting for an endoscopy is already treated with PPI before an endoscopy is even considered. To our knowledge this is the first non-interventional prospective study that specifically examined this cohort of patients and aimed to define the response to therapy and possible influence of socio-demographic and lifestyle factors in a real world clinical setting. It is very striking that only a very small proportion of patients experience a substantial improvement of symptoms after endoscopy and being treated with a PPI.

Whilst medication adherence could not be verified in this observational study, it is possible that all patients have taken the medication at the appropriate time or at the individually prescribed dosage. However, it seems unlikely poor compliance is the major explanation for this apparent failure of PPI, as they give rapid symptom relief[17]. We therefore hypothesize that in this patient group excess oesophageal acidification is not likely to be the major driver for the symptoms. It is likely that some of the symptoms are manifestation(s) of functional gastrointestinal disorders. These are known to commonly co-exist in subjects with reflux[18], and would substantially account for the lack of response to PPI therapy. On the other side it is interesting that 9% of the group reported not being offered PPI therapy despite having the cardinal symptoms of reflux (heartburn/regurgitation).

The marginal improvement in symptoms over time (SSS 7 at baseline and 5.5 at 6 mo) shows that the usual care approach adopted falls short with regard to improvement of symptoms when compared with trial data[16]. Altogether, less than one quarter of patients improved over 6 mo and the percentage of patients with moderate to severe symptom severity only decreased from 22% to 19% after 2 mo and was still 17% at 6 mo. This may reflect the fact that now predominantly non-responders to PPI therapy are referred for endoscopy. Our data may raise the question if other diagnostic and therapeutic approaches might be needed for these patients. Our results showed at baseline that higher LA grades were more likely found in men, older subjects, and participants with higher alcohol consumption, consistent with several studies[1,19,20]. Interestingly, whilst these factors are associated with more severe baseline reflux symptoms, they did not modify the symptomatic response to therapy, again suggesting that symptoms in our cohort may not be entirely attributable to GERD. This is likely to be due to our patient selection process due to biases inherent in their referral, which appears to have resulted in a group with "reflux symptoms" not due to clear-cut GERD (high proportion referred with ongoing symptoms despite PPI therapy).

Tobacco smoking is a listed as a major risk factor for many diseases, but there are few studies in regard to reflux symptoms. Due to lowering the pressure of the lower esophageal sphincter it is proposed as a possible risk factor for erosive esophagitis or Barrett's esophagus[9,21]. Our data however, revealed greater tobacco use

in patients with non-erosive or low grades esophagitis. This observation is based upon the *post-hoc* data analysis. Thus it needs to be independently confirmed before firm conclusions can be drawn with regard to this point. Of note, however, tobacco use did not influence the response to therapy over time in our cohort.

Whilst in the multivariable models, the set of socioeconomic and life style factors did not influence symptom severity over time, BMI > 30 kg/m^2, SES and employment status, as individual factors, did influence symptom severity in our population. Patients with a BMI > 30 kg/m^2 had greater improvement in SSS at both 2 and 6 mo, and advantaged social status and unemployment were both associated with a greater improvement in symptom severity over time. While this finding is based upon a *post-hoc* analysis and also requires independent prospective validation, it is reasonable to assume that a BMI > 30 kg/m^2 increases reflux of acidic content into the esophagus. Thus inhibiting acid secretion would reduce esophageal acid exposure and improve symptoms.

Our study has the strength that it reflects the routine clinical setting. Patients were studied with minimal interference. Thus our "real-life" setting did not reflect the setting of clinical trials with regular contacts that facilitate compliance with medication. While all patients had be informed and consent obtained at baseline and symptoms assessed during the follow-up, there were no other interferences with routine care that potentially could affect the outcome. While this provides insights into the real world, this must be balanced against some limitations including a possible participation bias by including only those choosing to complete the questionnaires, and the appreciable dropout rate after 2 mo. However, consistent with most clinical trials a considerable proportion of patients declined to participate or could not participate for various reasons. However, characteristics of patients included into the study were not different from patients not included. Thus our finding and conclusions appear to be relevant for the whole population. These are also part of the strength of the study as this is far more representative of what happens in real world clinical medicine.

In summary, contrasting general beliefs, the majority of patients with reflux symptoms referred for endoscopy continue to have symptoms in spite of the use of highly potent PPI's. Patients without endoscopic lesions appear to have more severe symptoms. The obvious persistence of symptoms suggests that there is the need to better monitor the response to therapy in these patients and to develop and properly use diagnostic and therapeutic strategies for patients with reflux symptoms who do not respond to the routine therapy with PPIs.

COMMENTS

Background

Authors aimed to study at 2 and 6 mo after endoscopy, the outcome of patients with symptoms of gastroesophageal reflux disease (GERD) referred for endoscopy under real life conditions. Little naturalistic data on reflux symptoms under usual care conditions exist. The majority of patients with GERD symptoms referred for endoscopy have already been or are currently treated with proton pump inhibitors (PPIs). In this setting symptoms are more severe in patients without erosive lesions. After endoscopy with continued PPI therapy, there is a significant but modest improvement of symptoms however, the majority of patients continue to have symptoms. Thus long-term PPI therapy appears to be insufficient in providing relief of symptoms in a considerable proportion of patients. Patients unresponsive to PPI therapy for reflux symptoms may benefit from investigations other than upper gastrointestinal endoscopy. There is a need to develop and test new approaches for these patients.

Research frontiers

Numerous trials suggest that in patients with gastro-oesophageal reflux disease the currently available treatments (*i.e.*, PPI) provide rapid control of symptoms and healing of lesions. Since these treatments are now widely available, they are frequently used prior to endoscopy and patients non-responsive to PPI are more likely to be referred for endoscopy. This study clearly demonstrates that there is a considerable unmet need with regard to symptom control in patients with gastro-oesophageal reflux disease.

Innovations and breakthroughs

This is the first study that prospectively assessed the symptoms of GERD patients' referred for endoscopy. The fact that the majority of patients continued to have symptoms contrasts general beliefs. Endoscopy alone might not be appropriate to target therapy in patients with GERD symptoms.

Applications

This research has considerable implications in the clinical setting. While highly potent treatments (*e.g.*, PPI) are widely available and used, patients referred for endoscopy are more likely to have symptoms that do not respond to PPI. Thus the study suggests that other treatment modalities might be required.

Peer review

In this manuscript, the authors investigated the outcome of patients with symptoms of GERD referred for endoscopy at 2 and 6 mo post endoscopy. The study was uniquely performed and the results were well discussed with no serious methodological issues.

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BRIEF ARTICLE

Trans-arterial chemo-embolization is safe and effective for very elderly patients with hepatocellular carcinoma

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Abstract

AIM: To assess the safety and efficacy of trans-arterial chemo-embolization (TACE) in very elderly patients.

METHODS: A prospective cohort study, from 2001 to 2010, compared clinical outcomes following TACE between patients ⩾ 75 years old and younger patients (aged between 65 and 75 years and younger than 65 years) with hepatocellular carcinoma (HCC), diagnosed according to the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases criteria. The decision that patients were not candidates for curative therapy was made by a multidisciplinary HCC team. Data collected included demographics, co-morbidities, liver disease etiology, liver disease severity and the number of procedures. The primary outcome was mortality; secondary outcomes included post-embolization syndrome (nausea, fever, abdominal right upper quadrant pain, increase in liver enzymes with no evidence of sepsis and with a clinical course limited to 3-4 d post procedure) and 30-d complications. Additionally, changes in liver enzyme measurements were assessed [alanine and aspartate aminotransferase (ALT and AST), gamma-glutamyl transpeptidase and alkaline phosphatase] in the week following TACE. Analysis employed both univariate and multivariate methods (Cox regression models).

RESULTS: Of 102 patients who underwent TACE as sole treatment, 10 patients (9.8%) were > 80 years old at diagnosis; 13 (12.7%) were between 75 and 80 years, 45 (44.1%) were between 65 and 75 years and 34 (33.3%) were younger than 65 years. Survival analysis demonstrated similar survival patterns between the elderly patients and younger patients. Age was also not associated with the adverse event rate. Survival rates at 1, 2 and 3 years from diagnosis were 74%, 37% and 31% among patients < 65 years; 83%, 66% and 48% among patients aged 65 to 75 years; and 86%, 41% and 23% among patients ⩾ 75 years. There were no differences between the age groups in the pre-procedural care, including preventive treatment for contrast nephropathy and prophylactic antibiotics. Multivariate survival analysis, controlling for disease stage at diagnosis with the Barcelona Clinic Liver Cancer score, number of TACE procedures, sex and alpha-fetoprotein level at the time of diagnosis, found no significant difference in the mortality hazard for elderly *vs* younger patients, and there were no differences in post-procedural complications. Serum creatinine levels did not change after 55% of the procedures, in all age groups. In 42% of all procedures, serum creatinine levels increased by no more than 25% above

the baseline levels prior to TACE. Overall, there were 69 post-embolization events (23%). Hepatocellular enzymes often increased following TACE, with no association with prognosis. In 40% of the procedures, ALT and AST levels rose by at least 100%. The increases in hepatocellular enzymes occurred similarly in all age groups.

CONCLUSION: TACE is safe and effective in very elderly patients with HCC, and is not associated with decreased survival or increased complication rates.

Key words: Hepatocellular carcinoma; Chemoembolization; Therapeutic; Elderly; Prognosis; Complications

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second cause of cancer-related deaths in men. It is the seventh most common cancer and sixth cause of cancer-related deaths among women[1]. The mean age of HCC diagnosis is increasing, and likewise the proportion of older patients seeking treatment for this deadly malignancy[2]. Latest estimates indicate that HCC incidence peaks above the age of 70 years[2,3].

Treatment modalities of HCC are limited. Curative treatment includes: liver resection, liver transplantation and, in small tumors, radiofrequency ablation (RFA). Only a small fraction of patients are eligible for these treatments. Most remaining patients receive palliative treatments including trans-arterial chemo-embolization (TACE), palliative RFA, sorafenib, or supportive care.

Compared to young patients with HCC, elderly patients have more co-morbidities and are considered poorer surgical candidates. Furthermore, patients older than 65-70 years of age are usually not considered to be potential candidates for liver transplantation[4]. Recently, sorafenib, the only proven chemotherapy to show efficacy against HCC, was evaluated in elderly patients (> 70 years), and was shown to have similar survival benefits as in patients younger than 70 years[5].

TACE has been previously shown to prolong survival among patients diagnosed with HCC who are not candidates for curative treatment. These data were recently challenged when a meta-analysis, using stringent inclusion and exclusion criteria, did not demonstrate a survival benefit for TACE[6]. One limitation of that study was that most of the data were derived from case series and clinical trials which included patients who were younger than 65 years[7,8].

There are only a few studies which have assessed the role of TACE among elderly patients. Most of these studies defined elderly as patients over 70 years of age, including a Chinese cohort of 196 patients older than 70 years[9] and an Italian cohort of 158 patients[10]. In both studies, the majority of elderly patients were between 70 and 75 years of age.

Since 2000, we have prospectively enrolled patients in our HCC database. We noticed that our population is aging and includes patients in their late seventies, eighties and even nineties, seeking treatment. Our aim was to assess efficacy, survival and safety of TACE among very elderly patients defined as ⩾ 75 years old, compared to younger patients in our cohort.

MATERIALS AND METHODS

Local institutional board approval was received for this study, with waiver of the need for informed consent as identifying data were omitted after data collection and there was no intervention performed (HMO 0604-10). All patients diagnosed with HCC between 2000 and 2010 who underwent TACE were included and prospectively followed until January 2012. HCC diagnosis was determined in accordance with established guidelines published by both the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases[11,12]. In cases where the radiological findings were inconclusive in establishing the diagnosis, percutaneous image-guided liver biopsy was performed. The decision that patients were not candidates for curative therapy was made by a multidisciplinary HCC team including the treating physician.

Demographic and clinical features were collected from patients' medical records. Patients were designated into three cohorts, stratified by age at diagnosis (< 65 years of age, 65-75 years of age, ⩾ 75 years of age). All patient data were collected using a national identification number.

TACE technique

Using a standard angiographic approach, transfemoral visceral arteriography was performed. Whenever possible, super selective TACE was attempted using a co-axial microcatheter and a mixture of 50 mg doxorubicin with lipiodol oil and gelfoam slurry or powder. If dictated by tumor burden and feasibility (hepatic reserve, Child Pugh status), segmental or lobar TACE was performed. Percutaneous vascular closure devices were routinely employed from 2007 onward.

Statistical analysis

All TACE procedures were recorded in the patient electronic records and all procedures were confirmed through the computerized hospital billing database. Inpatient notes and computer records were reviewed in order to extract clinical events, laboratory test results and medica-

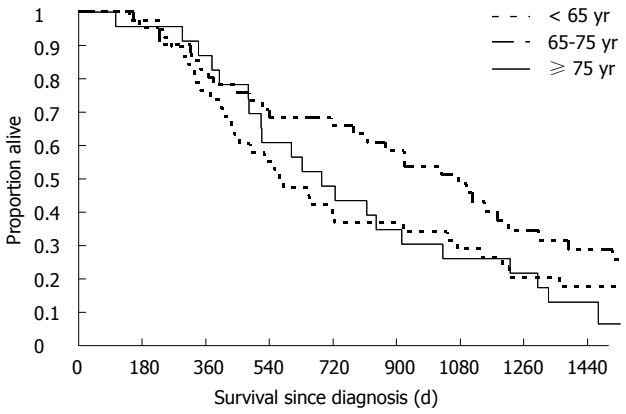

Figure 1 Survival curves comparing the age groups. Kaplan-Meier survival curves presenting the proportion of patients alive since hepatocellular diagnosis, stratified by age groups: < 65, 65-75 and ⩾ 75 years. The survival curves are not statistically different (log-rank $P = 0.19$).

tion provisions. The primary outcome of this analysis was overall mortality. Mortality rates and dates were determined *via* the national population registry. Secondary outcomes included post-embolization syndrome (nausea, fever, abdominal right upper quadrant pain, increase in liver enzymes with no evidence of sepsis and with a clinical course limited to 3-4 d post procedure); 30-d complications (sepsis, acute kidney injury, vascular events), as recorded in the medical charts; 30-d all-cause-readmission or all-cause-return to the emergency department. We also recorded pre-procedure (one week prior) and post-procedure (one week after) levels of creatinine, alanine and aspartate aminotransferase (ALT and AST), and gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALKP). We cross-referenced clinical impressions documented in the medical files by examining all medical documents available to reduce bias resulting from human error and ascertainment bias.

Descriptive statistics were employed to presented age group characteristics. Categorical variables are depicted with percentages and distributions, and continuous variables are presented with mean ± SD. Associations between categorical variables were assessed with the Fisher exact test, and comparison of continuous variables was performed with the Student's *t*-test, or with the Mann-Whitey *U* test. Survival charts were generated employing the Kaplan-Meier method, and survival curves were compared with the log-rank test. After confirming that the proportional hazard assumptions were met, we examined the association of age group with mortality using Cox proportional hazard regression analysis. Co-variables found on univariate analyses to have a seemingly probable association with mortality ($P < 0.1$) were included in the model. There were two types of missing data - treatment data and patient laboratory data. Treatments not specifically documented in the patient chart orders were considered not to have been provided (for example, preparation for contrast injection, antibiotic prophylaxis). In cases where laboratory tests were not taken, they were treated as missing and not included in the relevant analyses. In all analyses, two-tailed $P < 0.05$ was considered statistically significant. We examined whether absolute and relative differences between pre-procedure and post-procedure laboratory tests were different between the age groups using one-way analysis of variance and plotted the relationship between these measures.

RESULTS

Between 2000 and 2010, 235 patients were diagnosed with HCC. Of these, 102 patients were treated with TACE alone. Thirty-day follow-up was complete (all living patients were evaluated in the liver clinic outpatient service) and survival follow-up was complete (none of the patients left the country and the population registry is updated regularly). Data collection was complete for laboratory data, and all patient files were available for clinical assessment.

We divided our population into 3 cohorts: age < 65 years (group 1), age between 65 and 75 years (group 2) and age ⩾ 75 years (group 3). There were 38 patients in group 1, 41 patients in group 2 and 23 patients in group 3. Patient characteristics are presented in Table 1. There were 27 males and 75 females, with only three males in the younger age group. Younger patients had more advanced disease, as assessed by the Cancer of the Liver Italian Program (CLIP), Okuda or Barcelona Clinic Liver Cancer (BCLC) staging systems. The differences were less pronounced with the BCLC system, which accounts for functional status parameters, and we used this staging system to assess the primary outcome with multivariate analysis. Elderly patients had a mean alpha-fetoprotein (AFP) level at diagnosis that was higher than the two other age groups. The distribution of the number of TACE procedures per patient was similar between the age groups.

Survival analysis demonstrated similar survival patterns among the elderly patients and younger patients (Figure 1, $P = 0.19$). Overall, the cumulative follow-up time was 258 patient years. Median survival from diagnosis was 574 d (range: 143 d to 6 years), 1032 d (range: 154 d to 10 years) and 688 d (range: 104 d to 4.2 years) among patients in groups 1, 2 and 3, respectively. Respective survival rates at 1, 2 and 3 years from diagnosis were 74%, 37% and 31% among group 1 patients; 83%, 66% and 48% among group 2 patients; and 86%, 41% and 23% among group 3 patients. Multivariate survival analysis using the Cox proportional hazard regression model with variables of age (according to group), disease stage at diagnosis, number of TACE procedures, sex and AFP level at diagnosis found no significant difference in the mortality hazard of very elderly *vs* younger patients. The analysis was repeated with both the CLIP and Okuda staging systems, and the results were stable and consistent (Table 1).

Next, we assessed whether the number of TACE procedures was different among the groups. We hypothesized that elderly patients may have received different TACE regimens. The cohort patients described above under-

Table 1 Patient characteristics *n* (%)

	< 65 yr (*n* = 38)	65-75 yr (*n* = 41)	⩾ 75 yr (*n* = 23)	*P* value	HR (95%CI)
Age group multivariate HR (95%CI)[1]	1	1.03 (0.58-1.83)	1.04 (0.56-1.9)		-
Female	35 (92.2)	24 (58.6)	16 (69.6)	0.003	0.55 (0.31-0.98)[1]
Cirrhosis at diagnosis	35 (92.1)	41 (1)	22 (95.6)	0.338	
Ascites at diagnosis	4 (10.5)	4 (9.7)	4 (17.3)	0.632	
Hepatitis B virus	12 (31.5)	7 (17.0)	2 (8.6)	0.06	0.85 (0.49-1.47)[1]
Hepatitis C virus	21 (55.2)	29 (70.7)	18 (78.2)	0.14	
Cancer of the Liver Italian Program[2]				0.008	
0	8 (21.0)	20 (48.7)	7 (30.4)		1.9 (1.5-2.4)[2]
1	6 (15.7)	13 (31.7)	9 (39.1)		
2	17 (44.7)	8 (19.5)	5 (21.7)		
3	6 (15.7)	0 (0.0)	2 (8.6)		
4	1 (2.6)	0 (0.0)	0 (0.0)		
Okuda[2]				0.001	
1	21 (55.2)	39 (95.1)	19 (82.6)		5 (2.7-9.1)[2]
2	16 (42.1)	2 (4.8)	4 (17.3)		
3	1 (2.6)	0 (0.0)	0 (0.0)		
Child-Pugh-Turcot[2]				0.98	
1	31 (81.5)	35 (85.3)	19 (82.6)		2.1 (1.2-3.6)[2]
2	6 (15.7)	5 (12.1)	3 (13.0)		
3	1 (2.6)	1 (2.4)	1 (4.3)		
Barcelona Clinic Liver Cancer				0.027	
1	3 (7.8)	15 (36.5)	3 (13.0)		2.3 (1.65-3.2)[2]
2	7 (18.4)	6 (14.6)	5 (21.7)		
3	26 (68.4)	19 (46.3)	15 (65.2)		
Procedures				0.16	
1	12 (31.5)	4 (9.7)	7 (30.4)		
2	12 (31.5)	12 (29.2)	8 (34.7)		
3	6 (15.7)	11 (26.8)	2 (8.6)		
> 3	8 (20.9)	14 (31.4)	6 (26.0)		
Number of procedures	2.4 ± 1.6	3.4 ± 2.0	2.7 ± 2	0.07	
Albumin at diagnosis	36.37 ± 4.4	36.3 ± 4.7	36.0 ± 5.2	0.96	0.97 (0.84-1.11)[1]
Alpha-fetoprotein at diagnosis	944 ± 2162	337 ± 729	9232 ± 31376	0.05	
International normalized ratio at diagnosis	1.14 ± 0.23	1.19 ± 0.25	1.12 ± 0.18	0.46	1[1]
Bilirubin at diagnosis	2.9 ± 7.8	1.02 ± 0.66	1.17 ± 0.56	0.19	

[1]Hazard ratio (HR) (95%CI) when controlling for Barcelona Clinic Liver Cancer (BCLC) in multivariate model; [2]Multivariate HRs used instead of BCLC.

Table 2 Trans-arterial chemo-embolization procedure characteristics *n* (%)

	< 65 yr	65-75 yr	⩾ 75 yr	*P* value
Number of procedures	93	129	61	
Preparation for contrast material exposure	18 (19)	38 (29)	14 (22)	0.37
Iodine allergy and preparation	1 (1)	21 (16)	0 (0)	< 0.001
Antibiotic prophylaxis	68 (73)	95 (73)	44 (72)	0.7
Cefamezine	63 (67)	87 (67)	44 (71)	
Ceftazidime	1 (1)	1 (0.7)	0 (0)	
Clindamycin	2 (2)	1 (0.7)	0 (0)	
Clindamycin and Ciprofloxacin	0 (0)	1 (0.7)	0 (0)	
Vancomycin	2 (2)	1 (0.7)	0 (0)	
Right upper quadrant abdominal pain	16 (17)	24 (18)	5 (8)	0.22
Nausea	9 (9.6)	17 (13.1)	1 (1.6)	0.057
Fatigue	6 (6)	6 (4)	1 (1)	0.36
Fever	21 (22)	26 (2)	5 (8)	0.07
Post-embolization syndrome	23 (24)	36 (27)	10 (16)	0.3
Readmission	3 (3.2)	8 (6.2)	1 (1.6)	0.34
Total length of stay	3.6 (1.14)	3.55 (1.1)	3.3 (0.9)	0.19

went a total of 299 TACE procedures (Table 2). There were no differences between the age groups in pre-procedural care, including preventive treatment for contrast nephropathy and prophylactic antibiotics.

There were also no differences in post-procedural complications. There was a trend towards fewer post-embolization syndrome events among the elderly patients. Overall, there were 69 post-embolization syndrome events (23%). Some complications were very rare, including two cases of acute kidney injury (0.6%), two cases of sepsis (0.6%), two cases of hemorrhage (0.6%) and two cases of hemodynamic instability (0.6%). There was one event of vascular dissection and one event of pseudo-aneurysm.

There were 10 patients aged 80 years or older at diagnosis who were included in the analysis. Eight were women, two had BCLC stage Ⅰ, three had BCLC stage Ⅱ and five had BCLC stage Ⅲ. Eight were HCV carriers. These patients underwent 34 procedures; there were five post-embolization events (15%) and one readmission within 30 d of the procedure. Nine patients survived one year; of these, three survived two years, while two survived three years.

We hypothesized that older patients may be more prone to contrast-induced renal injury following TACE. Serum creatinine levels did not change after 55% (group 1), 58% (group 2) and 55% (group 3) of the procedures (P = 0.98). In 42% of all procedures, serum creatinine

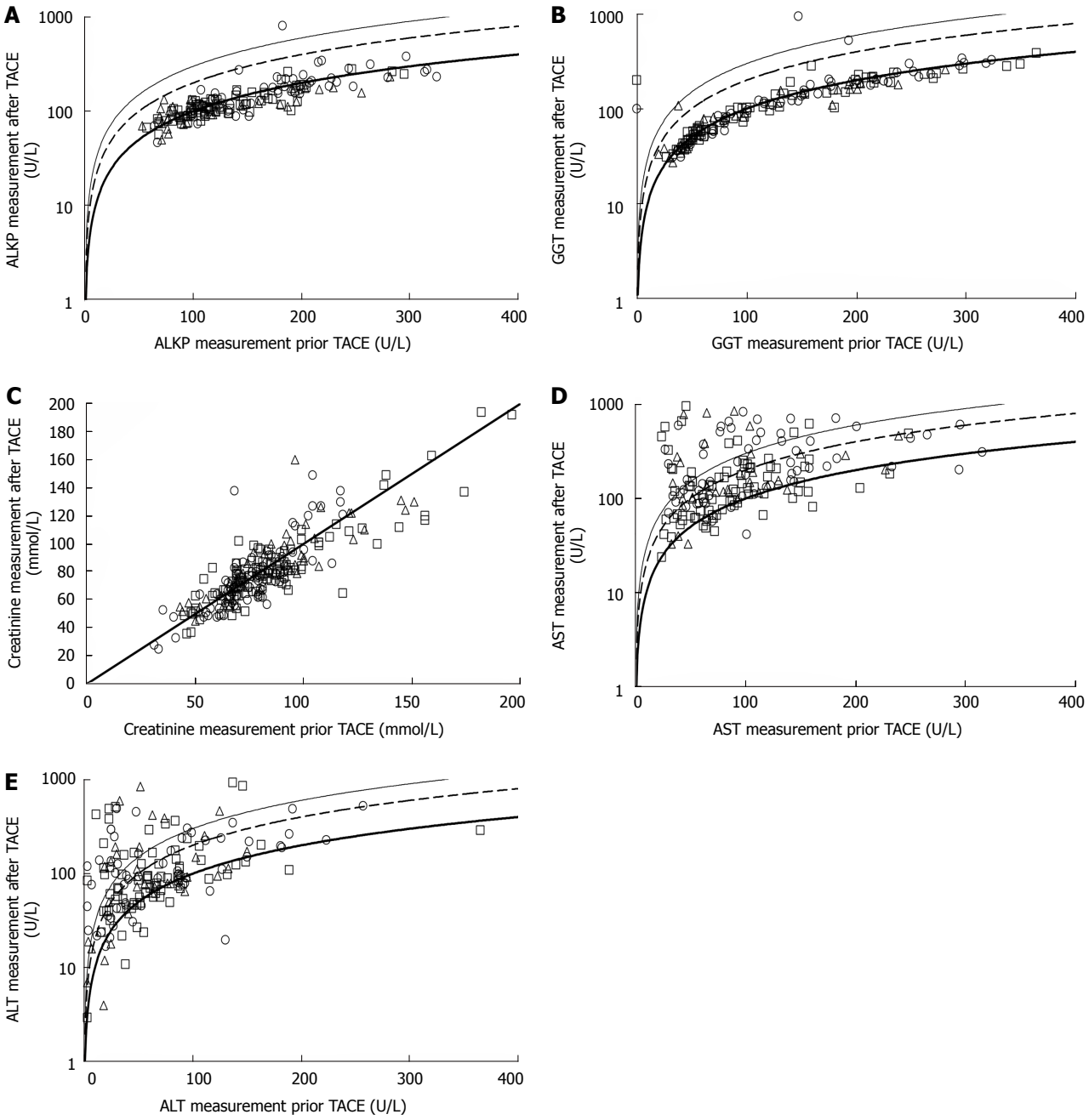

Figure 2 Laboratory results before and after trans-arterial chemoembolization procedures. A: Alkaline phosphatase (ALKP); B: Gamma-glutamyl transferase (GGT); C: Creatinine; D: Aspartate aminotransferase (AST); E: Alanine aminotransferase (ALT). Values before trans-arterial chemo-embolization (TACE) are in the x-axis, values after TACE are in the y-axis. In panels A, B, D and E, the vertical axis is in a logarithmic scale. In all panels the thick line represents y = x, the dashed line represents y = 2 × x and the thin line represents y = 3 × x. In all panels patients aged < 65 years are represented in circles, patients aged 65-75 years are presented with squares and patients aged ⩾ 75 years are represented with triangles.

levels increased by no more than 25% above the baseline measurement taken prior to TACE. There were only two cases in which creatinine more than doubled (Figure 2). Increases in creatinine levels were not associated with increased mortality.

Both ALT and AST levels frequently rose following TACE. These increases were evenly distributed among age groups (P = 0.17 for ALT, and P = 0.69 for AST). ALT and AST levels were not increased after 25% and 17% of TACE procedures, respectively. In contrast, ALT and AST more than doubled in 40% and 43% of the procedures, respectively (Figure 2). GGT and ALKP did not increase following TACE procedures in any of the age groups (Figure 2). There was no association between post-TACE rise in hepatocellular enzymes and mortality.

DISCUSSION

In this study, we show that TACE is safe and effective in very elderly patients (⩾ 75 years of age) who were diagnosed with HCC. Our results support previous findings which show that both survival and post-TACE

complications do not differ between older and younger patients.

Current guidelines for the management of HCC do not stratify strategies according to age[11]. Still, the treating clinician may be concerned that TACE will be more hazardous for elderly patients, due to the perceived increased chance of renal or vascular complications, or the potential for liver function deterioration. Furthermore, given that elderly patients have a shorter life expectancy, the treating physician may assume that elderly patients might not survive long enough to benefit from any potential gains that the TACE confers on younger patients.

Previous studies used different cutoffs to define elderly. Some even defined elderly patients as those above 60 or 65 years[13-17]. In a meta-analysis of randomized controlled trials, published in 2002, increased age was not associated with decreased prognosis. However, mean patient age in the included studies ranged from 41 to 66 years of age. In most of the included trials, more than 50% of the participating patients had a Child-Pugh score below 7[7]. In these cohorts there was a significant misrepresentation of the older patients, far less than their proportion among patients with HCC, suggesting marked selection bias[18].

Reports of treatment outcomes in patients older than 70 years have shown inconsistent results with regard to prognosis, although most have shown that advanced age is not associated with worse prognosis. Poon *et al*[19] have shown that among patients older than 70 years, those offered resection and those offered TACE had comparable prognosis with treatment-matched younger controls. In a recent report describing the role of TACE among patients with HCC excluded from transplantation or surgical resection, the mean age of the patients was 70 years, survival was not stratified by age and was estimated at 91%, 86% and 80% at 1, 2 and 3 years, respectively[20]. A similar study, with 95 elderly patients (defined as above 70 years old, though most were younger than 75 years) demonstrated that after excluding patients referred for transplantation, age was not associated with poorer survival. Survival rates among patients above 70 years of age were 51%, 36% and 23% at 1, 2 and 3 years, respectively[21].

The Italian Liver Cancer group compared treatments for HCC over a twenty-year period. They included a comparison of elderly and younger patients who underwent TACE with an age cutoff of 70 years and a mean age in the elderly group of 74.9 years. They report no difference in prognosis between elderly and younger patients who underwent TACE[10]. Another study from China presented similar results[9].

Only a few studies have included a larger proportion of older patients. Dohmen *et al*[22] analyzed a cohort of 36 patients with HCC who were older than 80 years, and showed that disease stage rather than age was the major determinant of survival. The patients in the study received various interventions including TACE, chemotherapy and surgical resection. Two other studies showed that patients older than 75 years with HCC had a worse prognosis than younger patients, but attributed these findings to poorer treatment rather than age effect[23,24]. An analysis that focused on all treatment modalities in 40 patients older than 75 years compared to younger patients showed no difference in survival rates. In that study, 43% of the younger controls underwent liver transplantation. Among the elderly, TACE was the most frequent treatment modality[25]. In 2010, a study of patients who underwent TACE, which included 131 patients aged 70 to 79 years and 69 patients older than 80 years, showed that age was a predictor of increased mortality[26].

We found a 23% (69/299) post-embolization syndrome rate and a 2.4% (12/299) hospital readmission rate in patients undergoing TACE. Both complications were not increased in elderly patients. A recent review of adverse events associated with TACE reported the incidence of hepatic insufficiency as ranging between 1% and 50%, and that of cholecystitis was between 0% and 10%[16]. Post-embolization syndrome rates have been reported between 2% and 80%[6,8]. Previous assessment of acute kidney injury among patients undergoing TACE found no association between this adverse event and age[26].

Because our study was conducted in a "real-life" setting, it suffers from selection bias. This limitation is shared by all previously published studies. The strengths of our study are the prospective design, with complete patient follow-up, record analysis and data acquisition. We use a clear age cut-off of 75 years to define elderly patients. An important strength of our study is the systematic documentation of post-embolization complications, segregated to the age groups, and also the presentation of biochemistry result dynamics.

Given the body of literature and the natural history of non-curable HCC, our study provides data to support the use of TACE in selected, very elderly patients (older than 75 years old). These patients should be offered similar treatment regimens, including TACE and palliative care, as younger patients. TACE should not be withheld from elderly patients based on age criteria alone.

COMMENTS

Background

Hepatocellular carcinoma (HCC) incidence is highest among patients over 70 years old. Though many are not candidates for curative therapy, they can benefit from life-prolonging interventions. Most existing literature provides information about younger patients. Trans-arterial chemo-embolization (TACE) has been shown to prolong survival among HCC patients, though there is little evidence of the procedure's safety and efficacy among very elderly HCC patients (above 75 years of age).

Research frontiers

HCC is one of the most common neoplastic malignancies worldwide. Therapeutic options aiming for cure include liver transplantation, liver resection and radiofrequency ablation (RFA). TACE, sorafenib, palliative RFA and supportive care are palliative options, which in some cases also offer life prolongation. Current clinical research challenges include: (1) Innovative discovery of novel therapeutic modalities; (2) Improving the use of known treatment options by identifying patient characteristics which can predict better outcomes, which therapeutic option will maximize outcome and to broaden the population eligible to receive

treatment. These challenges are met with an aging patient population, as the proportion of newly diagnosed elderly patients is consistently increasing.

Innovations and breakthroughs

In this study the authors included very elderly patients (older than 75 years), who have not been sufficiently represented in most published research so far. The authors compared survival time since diagnosis between the very elderly and younger patients. Additionally, the authors included critical information regarding TACE-associated complications and changes in common biochemistry tests. The authors show that the very elderly HCC patients enjoy the same survival benefits conferred by TACE on younger patients and that they do not experience more complications following this procedure.

Applications

There are several very practical implications which can be taken from this study: (1) TACE can be offered to patients above 75 years of age using the same clinical considerations which apply to younger patients; (2) Increases in hepatocellular enzymes alanine and aspartate aminotransferase are common and, by themselves, do not indicate poor or better response to treatment. In contrast, increases in the cholestatic enzymes gamma-glutamyl transpeptidase and alkaline phosphatase and in creatinine are not common and should warrant clinical investigation; (3) Post-embolization syndrome is common and has no prognostic implications.

Terminology

HCC is a malignant disease of the liver, often presenting as a complication of long-standing liver disease and cirrhosis due to viral, alcoholic and metabolic etiologies. TACE is a minimally invasive procedure in which an artery (most often the femoral artery) is punctured; through it a catheter is introduced and advanced towards the blood vessels which provide arterial blood to the liver. After identifying which specific vessels provide blood supply to the liver tumor, toxic chemotherapy is injected in order to cause cancer cell death, and additionally, the arteries are blocked (embolized) in order to stop the blood supply to the tumor.

Peer review

The study results are interesting and important with regard to epidemiological data. The theme is interesting and the study evaluations, as well as the statistical analysis, are well done. It is of great significance in providing evidence for clinicians to expand the age limit for TACE, and helps a lot for treatment decision making in elderly HCC patients.

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BRIEF ARTICLE

Effects of *Nigella sativa* on outcome of hepatitis C in Egypt

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Abstract

AIM: To evaluate the safety, efficacy and tolerability of *Nigella sativa* (*N. sativa*) in patients with hepatitis C not eligible for interferon (IFN)-α.

METHODS: Thirty patients with hepatitis C virus (HCV) infection, who were not eligible for IFN/ribavirin therapy, were included in the present study. Inclusion criteria included: patients with HCV with or without cirrhosis, who had a contraindication to IFN-α therapy, or had refused or had a financial constraint to IFN-α therapy. Exclusion criteria included: patients on IFN-α therapy, infection with hepatitis B or hepatitis I virus, hepatocellular carcinoma, other malignancies, major severe illness, or treatment non-compliance. Various parameters, including clinical parameters, complete blood count, liver function, renal function, plasma glucose, total antioxidant capacity (TAC), and polymerase chain reaction, were all assessed at baseline and at the end of the study. Clinical assessment included: hepato and/or splenomegaly, jaundice, palmar erythema, flapping tremors, spider naevi, lower-limb edema, and ascites. *N. sativa* was administered for three successive months at a dose of (450 mg three times daily). Clinical response and incidence of adverse drug reactions were assessed initially, periodically, and at the end of the study.

RESULTS: *N. sativa* administration significantly improved HCV viral load (380808.7 ± 610937 *vs* 147028.2 ± 475225.6, $P = 0.001$) and TAC (1.35 ± 0.5 *vs* 1.612 ± 0.56, $P = 0.001$). After *N. sativa* administration, the following laboratory parameters improved: total protein (7.1 ± 0.7 *vs* 7.5 ± 0.8, $P = 0.001$), albumin (3.5 ± 0.87 *vs* 3.69 ± 0.91, $P = 0.008$), red blood cell count (4.13 ± 0.9 *vs* 4.3 ± 0.9, $P = 0.001$), and platelet count (167.7 ± 91.2 *vs* 198.5 ± 103, $P = 0.004$). Fasting blood glucose (104.03 ± 43.42 *vs* 92.1 ± 31.34, $P = 0.001$) and postprandial blood glucose (143.67 ± 72.56 *vs* 112.1 ± 42.9, $P = 0.001$) were significantly decreased in both diabetic and non-diabetic HCV patients. Patients with lower-limb edema decreased significantly from baseline compared with after treatment [16 (53.30%) *vs* 7 (23.30%), $P = 0.004$]. Adverse drug reactions were unremarkable except for a few cases of epigastric pain and hypoglycemia that did not affect patient compliance.

CONCLUSION: *N. sativa* administration in patients with HCV was tolerable, safe, decreased viral load, and improved oxidative stress, clinical condition and glycemic control in diabetic patients.

Key words: Hepatitis C virus; *Nigella sativa*; Oxidative stress; Viral load

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INTRODUCTION

Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide (15%) and the highest prevalence of HCV-4 (67%) with a predominance of subtype 4a (55%)[1-4].

The natural history of HCV infection and disease progression are influenced by several factors such as age at infection onset, sex, duration of infection, co-infection with hepatitis B virus (HBV), level of HCV viremia and its genotype[5].

HCV is an important etiological factor for the development of hepatocellular carcinoma (HCC) and 23% of HCV patients develop HCC[6]. It has been shown that there is an alarming increase in the incidence of HCC in HCV patients in Egypt[7].

Presently, the only approved therapy for HCV is pegylated interferon-α (PEG-IFN-α) and ribavirin treatment, and their success is heavily influenced by patient adherence, which correlates directly with tolerance to their side effects[8]. Moreover, financial constraints for the combined therapy in many patients often contribute to therapy non-adherence, potentially lowering its success rates[9].

Oxidative-stress-related molecules may act as mediators modulating cellular events responsible for progression to liver fibrosis[10,11]. It has been shown that increased production of reactive oxygen species, in part catalyzed by iron overload, is involved in HCV-related liver damage through a pathway that involves DNA oxidative injury[12].

Silymarin is one of the alternative therapies that has been previously tested for the management of HCV patients who are not candidates for PEG-IFN; however, it has not shown any appreciable effects on viral load[13].

Nigella sativa (*N. sativa*) is used as a food condiment in the Middle East, and its seeds/oil have been shown to possess anti-inflammatory, antiviral and antineoplastic activity in various *in vitro* and *in vivo* studies[14]. The antioxidant effects of *N. sativa* have been shown in the essential oil obtained from six different extracts of its seeds, as well as from a commercial fixed oil[15]. The crude *N. sativa* oil and its fractions have shown potent *in vitro* radical scavenging activity[16].

The effect of *N. sativa* has been evaluated in animal studies. There are many reports of its biological activities including: immunopotentiation, antitumor, anti-inflammatory, analgesic, antihypertensive, antidiabetic, respiratory stimulation, antibacterial, antifungal, anticestode and antinematode effects[17-19].

A striking reduction of murine cytomegalovirus (CMV) virus titer in both spleen and liver was found in mice treated with *N. sativa* seed oil compared with control mice[20]. Moreover, oral feeding with *N. sativa* extract suppressed chemically induced hepatic tumors in rats[21]. *N. sativa* treatment has been shown to ameliorate disturbed hematological parameters in diabetic rabbits through modulation of lipid peroxide red blood cell (RBC) membrane content, leading to an increase in RBC count[22].

To date, no studies have addressed the use of *N. sativa* in HCV patients and its potential benefits; hence, we sought to evaluate the efficacy, safety, and tolerability of *N. sativa* supplementation as an alternative therapy in the management of HCV patients who are non-candidates for IFN-α therapy.

MATERIALS AND METHODS

This was a prospective, single-armed, self-controlled pilot study, conducted at the Tropical Medicine Department, El-Demerdash Hospital, Ain Shams University, Cairo, Egypt.

Patients

All HCV patients presenting to the department were assessed for eligibility. Inclusion criteria included all patients diagnosed with HCV with or without cirrhosis who either had a contraindication to IFN-α therapy[23], or had refused or had a financial constraint to IFN-α therapy. Exclusion criteria included: patients on IFN-α therapy; infection with HBV or hepatitis I virus; HCC or other malignancies; major severe illness such as renal failure, congestive heart failure, respiratory failure or autoimmune disease; or non-compliance to treatment. Informed consent was obtained from all patients, and the institutional ethical committee approved the study protocol, which conformed with the ethical guidelines of the 1975 Declaration of Helsinki.

Methods

Hepatitis markers were assessed for all patients at enrollment, including: hepatitis B core immunoglobulin G, hepatitis B surface antigen, and HCV antibody. All eligible patients were subjected to the following at enrollment and after 3 mo therapy: (1) Full clinical assessment with an emphasis on hepato- and/or splenomegaly, jaundice, palmar erythema, flapping tremors, spider naevi, lower-limb edema, and ascites; (2) Abdominal ultrasonography; (3) Laboratory investigations including complete blood count, liver functions [aspartate aminotransferase (AST), alanine aminotransferase (ALT), total proteins, albumin, total and direct bilirubin, prothrombin time and international standard ratio (INR)], renal function (serum creatinine, blood urea nitrogen), serum α-fetoprotein, polymerase chain reaction (PCR) for HCV (lower detection limit, < 50 copies) and total antioxidant capacity (TAC); (4) The antioxidants assessed in the estimation of TAC included enzymes such as superoxide dismutase, catalase, glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, ferritin; small molecules, including ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, and bilirubin; (5) The assay principle depended

Table 1 Clinical assessment data at baseline and after treatment *n* (%)

Characteristic	Baseline	After treatment	*P* value
Hepato and/or splenomegaly	19 (63.30)	19 (63.30)	
Jaundice	8 (26.70)	5 (16.70)	0.25
Palmar erythema	10 (33.30)	8 (26.70)	0.5
Spider naevi	8 (26.70)	4 (13.30)	0.125
Lower limb edema	16 (53.30)	7 (23.30)	0.004
Clinically detected ascites	13 (43.30)	8 (26.70)	0.063

After treatment: 3 mo *Nigella sativa* treatment. McNemar's test was used to compare categorical data overtime.

on the determination of the antioxidative capacity by the reaction of antioxidants in the sample with a defined amount of exogenously provide H_2O_2. The antioxidants in the sample eliminated a certain amount of the provided H_2O_2. The residual H_2O_2 was determined colorimetrically by an enzymatic reaction that involved the conversion of 3,5,dichloro-2-hydroxy benzensulfonate to a colored product; (6) TAC was analyzed using a TAC kit from Bio-diagnostic and measured spectrophotometrically using KENZA (Biolabo) analyzer; and (7) Real-time PCR was performed on COBAS TaqMan 48 PCR analyzer, using Roche COBAS Ampliprep Taqman Kit.

Drug administration

After performing the baseline evaluation, all patients received one capsule of *N. sativa* seed oil (450 mg) available as soft gelatin capsules (Baraka; Pharco Pharmaceuticals) three times daily after meals continuously for 3 mo. Patients were followed up every 2 wk throughout the study period for assessing treatment adherence, tolerability and incidence of adverse reactions.

Statistical analysis

Statistical analysis was performed using SPSS version 17 software. Numerical data were summarized using means and standard deviations or medians and ranges. Categorical data were summarized as percentages. Differences between numerical variables over two time measurements were tested using paired *t* test or medians test for non-normally distributed data. Repeated measures analysis of variance was used to test differences between three-time numerically normally distributed variables and Friedman test was used for non-normally distributed variables. McNemar's test was used to compare categorical data overtime. All *P* values were two-sided, and $P < 0.05$ was considered significant. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Thirty patients (16 male, 14 female) with a mean age of 47 ± 10.2 years fulfilled the inclusion criteria and were enrolled in the study. Four of those patients (13.33%) had diabetes and 26 (86.67%) did not. Fifteen patients (30%) had chronic liver disease, five (16.7%) had compensated cirrhosis, and 10 (33.3%) had decompensated cirrhosis.

Table 2 Laboratory data assessment at baseline and after treatment

Parameter	Base line	After 3 mo treatment	*P* value
Hemoglobin (g%)	11.8 ± 2.1	12.2 ± 2.2	0.1
RBCs (× 10^6/μL)	4.13 ± 0.9	4.3 ± 0.9	0.001
WBCs (× 10^3/μL)	6.4 ± 2.1	5.6 ± 2.2	0.013
Platelets (× 10^3/μL)	167.7 ± 91.2	198.5 ± 103	0.004
Hematocrit (%)	35.5 ± 6.3	37.3 ± 6.3	0.056
ALT (IU/L)	35.0 ± 15.7	41 ± 24.4	0.255
AST (IU/L)	40.9 ± 30.4	46.8 ± 32.2	0.307
Total protein (g/dL)	7.1 ± 0.7	7.5 ± 0.8	0.001
Albumin (g/dL)	3.5 ± 0.9	3.69 ± 0.9	0.008
Direct bilirubin (mg/dL)	0.5 ± 0.8	0.57 ± 1.5	0.745
Total bilirubin (mg/dL)	1.46 ± 1.5	1.36 ± 1.3	0.428
Prothrombin time (s)	14.1 ± 2.7	13.8 ± 2.2	0.562
INR	1.18 ± 0.2	1.2 ± 0.2	0.974
BUN (mg/dL)	13.5 ± 6.2	14.1 ± 5	0.540
Creatinin (mg/dL)	0.99 ± 0.4	0.88 ± 0.2	0.102
Serum AFP (IU/mL)	5.07 ± 1.8	4.67 ± 2.3	0.194
Sodium (mmole/L)	135.5 ± 6.1	133.5 ± 6	0.064
Potassium (mmole/L)	4.1 ± 0.5	4 ± 0.5	0.350
TAC (mmol/L)	1.35 ± 0.5	1.61 ± 0.6	0.001
Fasting blood sugar (mg/dL)	104.03 ± 43.4	92.1 ± 31.3	0.001
Post prandial blood sugar (mg/dL)	143.67 ± 72.6	112.1 ± 42.9	0.001
PCR (copies)	380808.7 ± 610937	147028.2 ± 475225.6	0.001

Paired *t*-test for all parameters, median test (equivalent to Wilcoxon matched pairs test) for polymerase chain reaction (PCR) levels. RBCs: Red blood cells; WBCs: White blood cells; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio; BUN: Blood urea nitrogen; AFP: α-fetoprotein; TAC: Total antioxidant capacity.

Patients' clinical assessment data before and after treatment are presented in Table 1. After treatment, there was a significant decrease in the percentage of patients with lower-limb edema, while there was no change in the percentage of patients with jaundice, palmar erythema, spider naevi or ascites. Laboratory parameters before and after treatment are presented in Table 2.

Liver functions tests

After 3 mo of *N. sativa* treatment, the mean HCV RNA levels (PCR) (147028.2 ± 475225.6) significantly decreased relative to their baseline levels (380808.7± 610937, $P = 0.001$) (Figure 1A). Table 3 presents the PCR responses after 3 mo treatment in patients with chronic liver disease and compensated and decompensated cirrhosis. Figure 2 presents individual patients' HCV RNA (PCR) values before and after treatment. Table 4 presents the Child-Pugh score and PCR response at baseline and after 3 mo in patients with compensated and decompensated cirrhosis. All cirrhotic patients (compensated and decompensated) showed no change or an improvement in their Child-Pugh score, patients presented with variable Child-Pugh score, yet the proportions' numbers were small for a valid statistical test. There was a significant increase in total

Table 3 Polymerase chain reaction response after treatment *n* (%)

Total responders	5 (16.67)
Chronic liver disease	3
Compensated cirrhosis	1
Decompensated cirrhosis	1
Total partial responders	15 (50)
Chronic liver disease	5
Compensated cirrhosis	4
Decreased 1 log	1
Decreased 2 log	3
Decompensated cirrhosis	6[1]
Total non-responders	10 (33.33)
Chronic liver disease	7
Compensated cirrhosis	
Decompensated cirrhosis	3

[1]Patients decreased polymerase chain reaction (PCR) but in same log. Non-responders: Patients did not show a decrease or showed an increase in PCR after 3 mo treatment with *Nigella sativa* (*N. sativa*); Responders: Patients became seronegative after 3 mo treatment with *N. sativa*; Partial responders: Patients showed a decrease in PCR but were still seropositive after 3 mo treatment with *N. sativa*.

Table 4 Child-Pugh score at baseline and after 3 mo in patients with compensated and decompensated cirrhosis

Patients	Child-Pugh score at baseline	Child-Pugh score after 3 mo of treatment	HCV RNA (PCR) response
1	B	B	Partial responder
2	C	B	Partial responder
3	A	A	Partial responder
4	B	B	Partial responder
5	A	A	Partial responder
6	C	C	Partial responder
7	C	B	Non- responder
8	C	B	Partial responder
9	A	A	Responder
10	B	B	Non-responder
11	C	B	Partial responder
12	B	A	Responder
13	C	B	Non-responder
14	A	A	Partial responder
15	A	A	Partial responder

HCV: Hepatitis C virus; PCR: Polymerase chain reaction.

protein and albumin levels after treatment. However, there was no significant change in liver enzymes (AST and ALT), bilirubin, or INR. Renal function did not show a significant change from baseline. TAC showed a significant increase after treatment (1.612 ± 0.56) relative to the baseline values (1.35 ± 0.05, $P = 0.001$, Figure 1B). Hematological functions varied significantly after 3 mo of *N. sativa* treatment. There was a significant increase in RBCs ($P = 0.001$) and platelets ($P = 0.004$) and a significant decrease ($P = 0.013$) in white blood cells.

Blood glucose

There was a significant decrease in both fasting and postprandial blood glucose after treatment ($P = 0.001$).

Incidence of side effects and drug interactions

The reported side effects throughout the study period were gastritis in one patient (3.33%) and hypoglycemia in five (16.76 %); of whom two had insulin-dependent diabetes, and the other three had advanced liver cirrhosis with possible glycogen depletion. Both side effects were treated and did not hinder completion of therapy. The only reported drug interaction was hypoglycemia due to concurrent use of insulin and *N. sativa*, which aggravated its hypoglycemic effects.

DISCUSSION

The main findings of our study were that administration of *N. sativa* significantly decreased HCV viral load, increased total antioxidant activity and total protein and albumin levels, lowered blood glucose levels, and improved lower-limb edema.

The anti-inflammatory, antiviral and antineoplastic activities of *N. sativa* have been previously documented in various *in vitro* and *in vivo* studies[14]. In the current study, *N. sativa* administration resulted in a significant decrease in viral load, with 16.67% of patients becoming seronegative, and 50% showing a significant decrease in the quantitative viral count. Among these, 66.7% had cirrhosis and 33.3% had chronic liver disease, implying antiviral activity. Patients with compensated and decompensated cirrhosis, either improved or maintained their baseline clinical condition and viral load, and none of them deteriorated, which signified the potential beneficial effects of *N. sativa* administration, as reflected by improvement in HCV RNA responses and clinical condition reflected in Child-Pugh class. Although the subcategory of cirrhosis patients was not large enough to detect significance, we recommend that larger studies should be conducted in patients with cirrhosis to confirm the potential beneficial effects offered by *N. sativa*, which might improve patients' overall outcome. To the best of our knowledge, this is the first human study to evaluate the effects of *N. sativa* on viral load in patients with HCV infection. Our findings of improved viral load could be explained by the results of a previous study of murine CMV[20], which showed a significant increase in macrophages and $CD4^+$ T cells, with a significant decrease in viral titer and increased serum IFN-γ levels in animals treated with *N. sativa*[24].

Oxidative-stress-related molecules have been shown to modulate cellular events responsible for the progression of liver fibrosis[10,11]. Moreover, HCV-related fibrosis, cirrhosis and liver failure have been found to be the result of an adaptive immune response to HCV-infected cells[25], which is mediated by induction of endoplasmic reticulum and oxidative stress and downregulation of antiapoptotic proteins nuclear factor-κB and Bcl-xl in infected hepatocytes[26].

In our study, *N. sativa* administration significantly increased TAC in HCV patients, implying the potential protective effect of *N. sativa* by halting the oxidative stress that contributes to disease progression. Further-

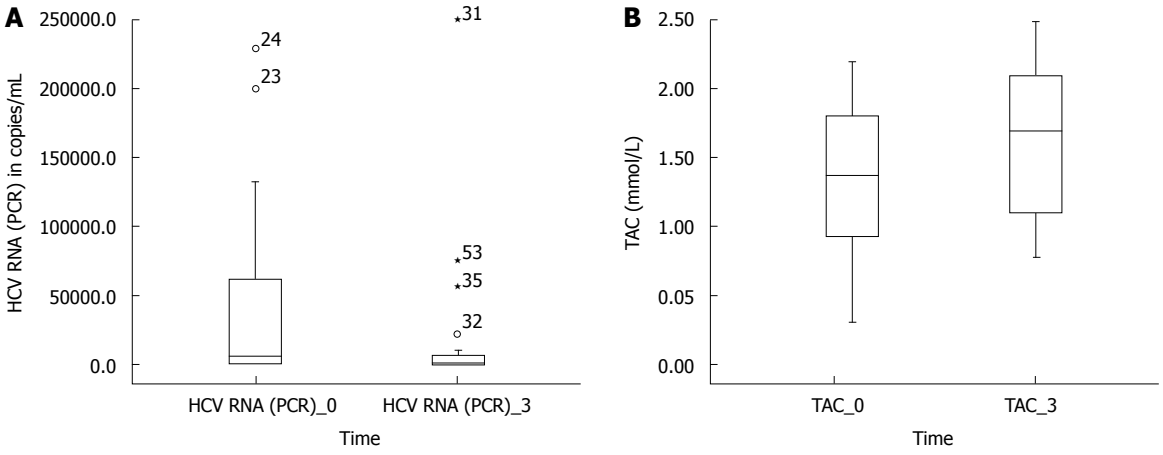

Figure 1 Box plot for hepatitis C virus RNA (polymerase chain reaction) levels (A), total antioxidant capacity (B) before and after treatment. A: Median test (equivalent to Wilcoxon matched pairs test), $P < 0.001$. Hepatitis C virus (HCV) RNA [(polymerase chain reaction (PCR)]_0: PCR values of patients before treatment; HCV RNA (PCR)_3: PCR values of patients after 3 mo treatment; B: Paired *t* test, $P < 0.001$. Total antioxidant capacity (TAC)_0: TAC levels of patients before treatment; TAC_3: TAC levels of patients after 3 mo treatment.

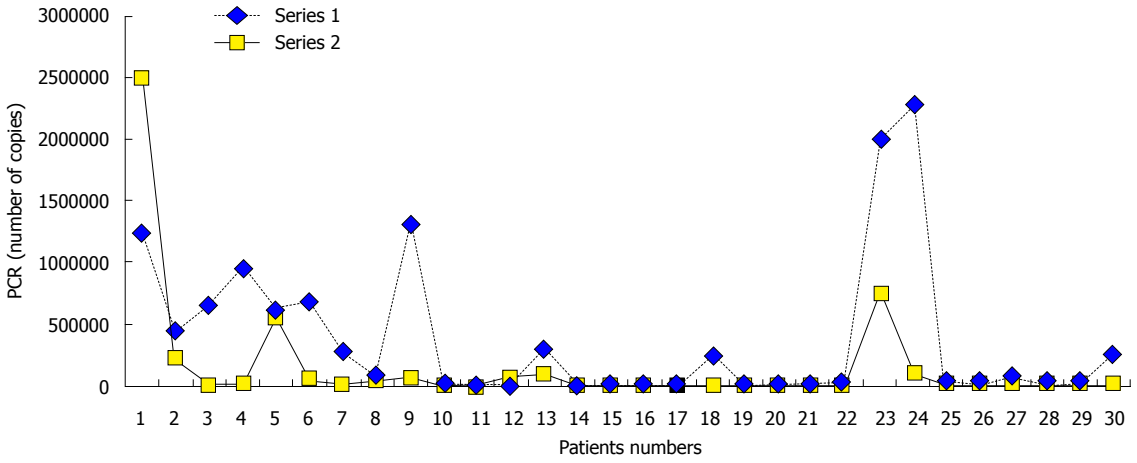

Figure 2 Line plot for polymerase chain reaction levels in individual patients at baseline and after 3 mo of treatment. Series 1: Polymerase chain reaction (PCR) values in all patients at baseline; Series 2: PCR values in all patients after 3 mo of treatment.

more, it is tempting to propose that increasing antioxidant capacity, with its cytoprotective role, contributed to decreasing the viral load.

The antioxidant effects of *N. sativa* have been previously elaborated in animal models of liver ischemia, in which it improved the antioxidant capacity and reduced oxidative stress[27]. Moreover, *N. sativa* increased hepatic glutathione and reduced elevated hepatic serum enzymes in carbon-tetrachloride-treated mice, ameliorating its hepatotoxic potential[15,28].

Some patients with acute and chronic liver disease develop diabetes mellitus[29,30]. HCV infection may also contribute to the development of diabetes, which has been observed in 21% of HCV-infected patients[31], and glucose intolerance has been seen in patients with HCV infection, compared with controls with liver diseases[32-35].

Insulin resistance is one of the pathological features in patients with HCV infection that may be associated with life-threatening complications, making HCV-associated insulin resistance a therapeutic target at any stage of HCV infection[36].

Our study showed that *N. sativa* treatment significantly decreased blood glucose levels in HCV patients, implying that it might offer a potential modulatory effect on HCV-induced glucose intolerance. This effect was beneficial in the control of diabetes in HCV patients because it allowed us to lower the insulin requirement. Similar results have been previously shown in a study of patients with diabetes, in whom administration of *N. sativa* (2 g/d) caused significant reductions in fasting blood glucose and 2-h postprandial blood glucose and hemoglobin A1c, and improved insulin resistance[37].

HCV infection itself can induce autoimmune hemolytic anemia, leukopenia, and thrombocytopenia, even in the absence of IFN-α treatment[38-42]. Hematopoietic growth factors modulating these complications have shown a beneficial role in HCV patients[43].

N. sativa therapy in our study significantly improved RBC and platelet counts in HCV patients, indicating a potential amelioration/prevention of HCV-induced

hematological disorders. Hence, *N. sativa* may positively affect clinical outcome in HCV patients.

The ability of *N. sativa* to improve hematological indices has also been reported in animal studies in which it increased both the packed cell volume and hemoglobin in treated rats[18], as well as increased RBC count in diabetic rabbits[44]. The increased RBC count was attributed to lowering of the membrane lipid peroxide level, leading to decreased susceptibility to hemolysis.

Serum albumin is the most abundant plasma protein[45] and is essential for maintaining oncotic pressure of the vascular system[46]. Chronic HCV patients may suffer a decrease in serum albumin level[47], and improvement in hypoalbuminemia has been shown to improve prognosis[48] and quality of life[49]. Concentrations of < 30 g/L were associated with an 85% chance of liver-related complications at 5 years and a 3-year mortality of 70%[50], and was predictive of morbidity and mortality in patients with liver cirrhosis[51,52].

In the current study, *N. sativa* administration significantly increased serum albumin levels and significantly reduced lower-limb edema, indicating an improvement in clinical condition. Prior animal studies have shown similar effects in rats[53] and broiler chickens[54] in a dose-dependent manner[55].

N. sativa is used in Arab folk medicine as a diuretic plant[56], the mechanism that can also contribute to its efficacy in decreasing lower limb edema, and its resolution in many patients.

In our study, the number of patients with ascites decreased after treatment with *N. sativa*, although the change was not significant; nevertheless, the change in ascites severity could not be totally denied, because the degree of ascites was not assessed sonographically. We hence recommend assessment of ascites incidence and severity in future studies to confirm these results.

The safety and tolerability of *N. sativa* have been previously documented in various clinical trials[57-60]. However, to date, clinical studies addressing *N. sativa* efficacy, safety and tolerability in HCV patients are lacking. Our study has shown that *N. sativa* was tolerable in all patients, and the only side effects reported were one patient with epigastric pain that was controlled with antacids, and five patients with hypoglycemia, two of whom had diabetes and were receiving concomitant insulin and the hypoglycemia did not recur after decreasing the insulin dose. Of note, the dose of *N. sativa* used in the current study was (1.35 g/d), which was slightly lower than in the other studies - 2 g/d used by Bamosa *et al*[37] - because this dose was available in the Egyptian market and was close to the doses previously used. Although *N. sativa* in such patients had significantly positive effects on many parameters, perhaps higher doses or longer durations of therapy may accentuate such appreciable effects. Further studies are needed to confirm such findings.

It can therefore be concluded that *N. sativa* administration can have a potential beneficial effect on HCV disease progression and outcome through its prominent antiviral, antioxidant and immunomodulatory effects and can minimize HCV-related hematological complications.

Our study had some limitations. This was the first clinical study to be performed in HCV patients and larger studies are required to confirm the results of the current study. We did not assess all patients for the amount of ascites after therapy sonographically, because such a favorable effect of *N. sativa* was not anticipated. Hence, in view of significant improvement of serum albumin, this effect of *N. sativa* on the amount of ascites needs further study. Liver biopsy was not performed, because the patients were either not eligible or refused the procedure.

In conclusion, *N. sativa* administration in HCV patients is safe and tolerable and results in a significant improvement in viral load, oxidative stress and laboratory markers. Moreover, the clinical improvement and better glycemic control in patients with diabetes indicate a potential role for *N. sativa* in improving the clinical outcome of HCV patients. We recommend larger controlled multicenter randomized studies for longer periods for evaluation of the potential beneficial role of *N. sativa* in HCV patients with and without concurrent IFN therapy.

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COMMENTS

Background

Hepatitis C virus (HCV) is an important etiological factor for the development of hepatocellular carcinoma. Pegylated interferon-α (PEG-IFN-α) and ribavirin treatment are the only currently approved therapy for HCV with variable response rate, and a success that is heavily influenced by patients' response rate, adherence to treatment, and tolerance to side effects. Moreover, the financial constraints for the combined therapy in many patients often contribute to their non-adherence to therapy, potentially lowering its success rates. *Nigella sativa* (*N. sativa*), a food condiment used in the Middle East, has shown anti-inflammatory, antiviral, antioxidant and anticancer activities in various *in vitro* and *in vivo* studies. To date, no studies have addressed the use of *N. sativa* in HCV patients and its potential benefits.

Research frontiers

N. sativa is a natural food supplement, and has shown beneficial antioxidant, antiviral, anticancer and immunopotentiating properties in various *in vitro* and *in vivo* studies, but HCV studies are lacking. In exploring the potential role of *N. sativa* in improving HCV patients' clinical outcome, the research hot spot is its beneficial effects on reducing viral load, improving antioxidant capacity, alleviating hematological parameters, and improving blood glucose control, especially in diabetes. All of which could have a potential beneficial effect on HCV patients' responses and amelioration of HCV-related complications.

Innovations and breakthroughs

No prior clinical trials in HCV patients have evaluated the use of *N. sativa* and its potential beneficial effects. No studies have addressed any alternative treatments for IFN non-eligible patients or those who refuse or cannot tolerate IFN therapy. *N. sativa* offers hope for a safe tolerable alternative to those patients who cannot tolerate IFN or have a contraindication to its use. Moreover, *N. sativa* has a potential benefit in improving clinical outcome. It showed a preliminary improvement in viral load and antioxidant levels that could provide a potential cure for HCV infection. *N. sativa* also improved the hematological profile and to-

tal protein and albumin levels, which contribute to HCV-induced complications. Moreover, *N. sativa* decreased blood glucose levels, and hence decreased insulin requirement in patients with diabetes.

Applications

The study results suggest that *N. sativa* is a potentially beneficial, safe and tolerable alternative in IFN non-eligible HCV patients. It can improve clinical outcome, ameliorate HCV-induced hematological and diabetic complications, and improve lower-limb edema.

Terminology

Viral load, also known as viral burden or viral titer, is a measure of the severity of a viral infection, and can be calculated by estimating the amount of virus in an involved body fluid, for example, RNA copies/mL blood plasma. Human serum albumin is the most abundant protein in human blood plasma. It is produced in the liver and constitutes about half of the blood serum protein. It transports hormones, fatty acids, and other compounds, buffers pH, and maintains osmotic pressure, among other functions. Total antioxidant capacity measures collectively the amount of antioxidant components of the body that reflects the body's capacity to combat oxidative stress.

Peer review

This was an interesting study in which the authors treated HCV patients with *N. sativa*, a food condiment used in the Middle East.

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BRIEF ARTICLE

ABO blood type, long-standing diabetes, and the risk of pancreatic cancer

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Abstract

AIM: To retrospectively study pancreatic cancer patients with respect to their ABO blood type and diabetes.

METHODS: Our analysis included a cohort of 1017 patients with pancreatic ductal cancer diagnosed at our hospital in Tokyo. They were divided into two groups: 114 patients with long-standing type 2 diabetes (DM group, defined as diabetes lasting for at least three years before the diagnosis of pancreatic cancer) and 903 patients without diabetes (non-DM group). Multivariate analysis was performed to identify factors that are associated with long-standing diabetes. The DM group was further divided into three subgroups according to the duration of diabetes (3-5 years, 5.1-14.9 years, and 15 years or more) and univariate analyses were performed.

RESULTS: Of the 883 pancreatic cancer patients with serologically assessed ABO blood type, 217 (24.6%) had blood type O. Compared with the non-DM group, the DM group had a higher frequency of blood type B [odds ratio (OR) = 2.61, 95%CI: 1.24-5.47; reference group: blood type A]. Moreover, male (OR = 3.17, 95%CI: 1.67-6.06), older than 70 years of age (OR = 2.19, 95%CI: 1.20-3.98) and presence of a family history of diabetes (OR = 6.21, 95%CI: 3.38-11.36) were associated with long-standing type 2 diabetes. The mean ages were 64.8 ± 9.2 years, 67.1 ± 9.8 years, and 71.7 ± 7.0 years in the subgroups with the duration of diabetes, 3-5 years, 5.1-14.9 years, and 15 years or more, respectively (P = 0.007). A comparison of ABO blood type distribution among the subgroups also showed a significant difference (P = 0.03).

CONCLUSION: The association of pancreatic cancer with blood type and duration of diabetes needs to be further examined in prospective studies.

Key words: Pancreatic cancer; ABO blood type; Diabetes mellitus; Risk factor; Screening

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INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer deaths in Japan, accounting for approximately 26000 deaths each year[1]. Because of the poor prognosis, identifying high-risk individuals and the modifying risk factors are important strategies for preventing pancreatic cancer. Despite intensive research efforts, the etiology of spo-

radic pancreatic cancer remains largely unknown. Epidemiologic studies have consistently shown that smoking and long-standing type 2 diabetes are two modifiable risk factors for pancreatic cancer[2,3]. The association between diabetes and pancreatic cancer is complex because diabetes and pancreatic cancer development may involve a similar pathogenesis and share common risk factors, such as obesity, smoking and insulin resistance. Moreover, although long-standing type 2 diabetes is a risk factor for pancreatic cancer, new-onset diabetes may also result from pancreatic cancer[4,5]. There is a lack of data on the proportion of pancreatic cancer cases that can be attributed to long-standing diabetes and the prevalence of pancreatic cancer-induced new-onset diabetes.

In addition, although an association between the ABO blood type and various diseases was proposed 50 years ago[6,7], the ABO blood type has recently been confirmed to be associated with malignant tumors, including skin cancer[8], esophageal cancer[9], hepatocellular carcinoma[10] and pancreatic cancer[11-16]. Regarding pancreatic cancer risk, both epidemiologic[11-15] and genome-wide association studies (GWAS)[16] showed that individuals carrying the O blood type had the lowest risk compared with those with non-O blood types.

Although diabetic patients may represent a high-risk group for pancreatic cancer, the increasing prevalence of type 2 diabetes in the general population and the lack of specific biomarkers do not justify screening all diabetic patients for the early detection of pancreatic cancer. It is possible that among diabetic patients, a subset of diabetics who are at high risk of developing pancreatic cancer may show different characteristics from other diabetics, including the duration of diabetes and blood type distribution. In this study, we retrospectively examined 1017 patients with pancreatic cancer, focusing on the duration of type 2 diabetes and the ABO blood type.

MATERIALS AND METHODS

Patients

We reviewed the medical records of patients with pancreatic ductal cancer diagnosed between 1975 and 2009 at Tokyo Metropolitan Komagome Hospital. A total of 1022 patients were included in the present analysis. Overall, 66.3% had histological confirmation, and the remaining patients were diagnosed based on either endoscopic retrograde cholangiopancreatography or at least two imaging modalities. To exclude the possibility that new-onset diabetes was caused by pancreatic cancer, we defined individuals with long-standing diabetes as those who had diabetes for at least 3 years before the diagnosis of pancreatic cancer. Among the 1022 patients, we excluded 5 patients with long-standing diabetes due to diagnoses other than type 2 diabetes.

The subjects were divided into two groups: 114 patients with long-standing type 2 diabetes (DM group) and 903 patients without long-standing type 2 diabetes (non-DM group). Furthermore, we classified the DM group into 3 subgroups according to the duration of preexisting diabetes: a relatively short period of 3-5 years (DM-S group: 31 patients), a medium range of 5.1-14.9 years (DM-M group: 48 patients), and a relatively long period of 15 years or more (DM-L group: 35 patients). Information on gender, age, smoking status, ABO blood type, diabetes, a family history of diabetes and tumor location was recorded from medical charts. The ABO blood type was assessed serologically, and the information of DM was primarily based on self-report. For 92 patients in the DM group, their medical history revealed the type of medical treatment that they had received for diabetes.

This study was approved by the Institutional Review Board of Tokyo Metropolitan Komagome Hospital.

Statistical analysis

First, age, gender, smoking status (never *vs* former or current), a family history of diabetes (present *vs* absent in a first-degree relative), the location of the cancer (head *vs* body or tail) and the ABO blood type were compared using univariate analysis. A two-sample *t*-test was conducted with age as a continuous variable. A χ^2 test was used for categorical variables. The unconditional logistic regression method was used to compare the DM group with the non-DM group using variables that showed a *P* value of less than 0.15 in the univariate analyses. Variables for which the *P* value exceeded 0.05 were eliminated in a stepwise fashion such that only those that had a statistically significant association with long-standing type 2 diabetes were included in the final regression model. In this analysis, blood type A was used as a reference group. The final models were evaluated for goodness-of-fit with the Hosmer-Lemeshow test.

Similar to the analyses mentioned above, we performed univariate analyses among 3 subgroups of the DM group. A one-way analysis of variance was conducted for continuous variables, and a χ^2 test was used for categorical variables.

We used the χ^2 test to compare the ABO blood type distribution in our pancreatic cancer patients with the distribution reported from a nationally representative sample of the Japanese population[17].

All of the *P* values were two-sided, with statistical significance set at $P < 0.05$. All of the statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) 19 statistical package software (IBM Japan, Tokyo).

RESULTS

Table 1 shows the characteristics of the DM group and the non-DM group and the results of univariate analyses and multivariate analysis. The sex ratio (male/female) was significantly higher in the DM group than in the non-DM group ($P = 0.002$). The mean age of the DM group was 1.8 years older at the diagnosis of pancreatic cancer than the non-DM group (67.9 ± 9.2 years *vs* 66.1 ± 10.6 years, $P = 0.08$). In accordance with this result, there were more patients older than 70 years of age

Table 1 Characteristics of the diabetes mellitus group and the non-diabetes mellitus group *n* (%)

Variables	DM group (*n* = 114)	Non-DM group (*n* = 903)	*P* value	OR (95%CI)
Sex			0.002	
Female	33 (28.9)	398 (44.1)		Reference
Male	81 (71.1)	505 (55.9)		3.17 (1.67-6.06)
Age (yr)			0.009	
< 70	53 (46.5)	541 (59.9)		Reference
⩾ 70	61 (53.5)	362 (40.1)		2.19 (1.20-3.98)
Smoking status	*n* = 99[1]	*n* = 715	0.28	
Former and current smokers	60 (60.6)	392 (54.8)		
Non-smokers	39 (39.4)	323 (45.2)		
Tumor location			0.37	
Head	58 (50.9)	501 (55.5)		
Body/tail	56 (49.1)	402 (44.5)		
Family history of DM	*n* = 76	*n* = 393	< 0.001	
No	41 (53.9)	335 (85.2)		Reference
Yes	35 (46.1)	58 (14.8)		6.21 (3.38-11.36)
ABO blood type	*n* = 104	*n* = 779	0.06	
A	35 (33.7)	338 (43.4)		Reference
B	28 (26.9)	175 (22.5)		2.61 (1.24-5.47)
O	34 (32.7)	183 (23.5)		1.92 (0.95-3.87)
AB	7 (6.7)	83 (10.7)		0.94 (0.28-3.14)

[1]Because of missing data, the numbers of subjects are presented for smoking status, family history of diabetes, and ABO blood type in each group. Diabetes mellitus (DM) group represents pancreatic cancer patients with long-standing type 2 diabetes, defined as diabetes lasting at 3 years prior to the diagnosis of pancreas cancer. Non-DM group represents pancreatic cancer patients without long-standing type 2 diabetes. OR: Odds ratio determined by the logistic regression method.

in the DM group than the non-DM group ($P = 0.009$). Subjects in the DM group were more likely to have a family history of diabetes than those in the non-DM group ($P < 0.001$). The distribution of the ABO blood type seemed to differ between the DM group and non-DM group ($P = 0.06$). There were no significant differences in the smoking status ($P = 0.28$) or tumor locations ($P = 0.37$) between the two groups.

The logistic regression method using candidate variables resulting from the univariate analyses revealed that sex, age, a family history of diabetes and ABO blood type were associated with long-standing type 2 diabetes. Interestingly, the frequency of blood type B was significantly higher in the DM group than in the non-DM group (Table 1). The Hosmer-Lemeshow test showed that the regression model had an acceptable goodness-of-fit ($P > 0.05$).

There were no significant differences in gender, smoking status, a family history of diabetes or the location of cancer among the 3 subgroups defined by the duration of diabetes. However, significant differences in age ($P = 0.007$) and the ABO blood type ($P = 0.03$) were observed among the 3 subgroups (Table 2). We found a significant difference in the ABO blood type distribution between our pancreatic cancer patients and the general Japanese population[17] ($P = 0.02$). As shown in Table 3, our patients had a lower frequency of blood type O and a higher frequency of blood type A.

Table 2 Comparison of characteristics among 3 diabetes mellitus subgroups according to duration of diabetes

Variables	DM-S (*n* = 31)	DM-M (*n* = 48)	DM-L (*n* = 35)	*P* value
Sex ratio (M/F)	2.44	2.69	2.18	0.91
Age, yr (mean ± SD)	64.8 ± 9.2	67.1 ± 9.8	71.7 ± 7.0	0.007
⩾ 70	41.9%	47.9%	71.4%	0.03
Smoking status	*n* = 26[1]	*n* = 43	*n* = 30	0.12
Former and current smokers	50.0%	72.1%	53.3%	
Tumor location: Head	54.8%	47.9%	51.4%	0.83
Family history of DM	*n* = 16	*n* = 36	*n* = 24	0.64
Positive family history of DM	56.3%	44.4%	41.7%	
ABO blood type	*n* = 26	*n* = 44	*n* = 34	0.03
A	19.2%	52.3%	20.6%	
B	26.9%	18.2%	38.2%	
O	42.3%	27.3%	32.4%	
AB	11.5%	2.3%	8.8%	

[1]Because of missing data, the numbers of subjects are presented for smoking status, family history of diabetes and ABO blood type in each group. F: Female; M: Male; DM: Diabetes mellitus; DM-S: Patients with diabetes of 3-5 years; DM-M: Patients with diabetes of 5.1-14.9 years; DM-L: Patients with diabetes of 15 years or more.

Table 3 Comparison of the distribution of ABO blood type between our cases and the general Japanese population *n* (%)

ABO blood type	Our pancreatic cancer patients (*n* = 883)	General Japanese population (*n* = 4465349)[1]
A	373 (42.2)	1725950 (38.7)
B	203 (23.0)	988996 (22.2)
O	217 (24.6)	1305924 (29.3)
AB	90 (10.2)	444479 (10.0)

[1]The data was referred to Fujitas' article[17].

DISCUSSION

In our retrospective examination of 1017 pancreatic cancer patients, we found that the distribution of the ABO blood type in our cases is different from that of the general Japanese population. Furthermore, the distribution of the blood type also seemed to differ between the DM group and the non-DM group, with the DM group having a higher frequency of blood type B. This finding suggests that long-standing type 2 diabetes and other underlying factors that are associated with diabetes, such as blood type, might play a role in predisposing diabetic patients to pancreatic cancer.

Because of the increasing prevalence of type 2 diabetes in the general population and the absence of specific biomarkers, it is not cost-effective to screen for pancreatic cancer in asymptomatic diabetics. Therefore, it is important to identify a subset of diabetics with a higher susceptibility to pancreatic cancer than other diabetics. We addressed this issue by focusing on the duration of diabetes and the ABO blood type.

It remains unclear whether the duration of diabetes significantly predicts pancreatic cancer risk. Previous studies have noted an inverse association between the duration of diabetes and the pancreatic cancer risk; the

association appeared to be strongest among individuals with a duration of diabetes less than 4 years, with a relative risk of 2.1 (95%CI: 1.9-2.3)[3]. However, in a large Korean cohort study, the pancreatic cancer risk was significantly increased with an increasing duration of diabetes in men: the hazard ratios were 2.0, 2.4 and 3.0 for individuals with a duration of diabetes less than 4.9 years, 5.0-9.9 years, and 10 years or more, respectively[18]. Despite the inverse association observed in a meta-analysis published in 2005, individuals with long-standing diabetes (> 5 years) were still at a 50% increased risk of pancreatic cancer[3]. Interestingly, when we divided the DM group into 3 subgroups according to the duration of diabetes, we found that among long-standing diabetes-related pancreatic cancer cases, there may be several subgroups that are associated with a specific blood type and characterized by the period from the onset of diabetes to the occurrence of pancreatic cancer. This finding suggests that patients with long-standing type 2 diabetes might not be considered a single uniform group.

Regarding the ABO blood type, several lines of evidence in recent years have shown that the ABO blood type is associated with a risk of pancreatic cancer. A prospective cohort study noted an elevated risk of incidental pancreatic cancer among subjects with blood type A, AB or B compared with blood type O, and those with blood type B had the highest risk[11]. In addition, they also reported increased risk with the addition of each non-O allele[12]. A recent GWAS, which mainly involved Caucasian populations, identified an association between a single-nucleotide polymorphism (SNP) in the ABO gene locus (rs505922) and pancreatic cancer[16]. Accordingly, an article by Nakao and co-workers, which is the only study on pancreatic cancer and the ABO blood type alleles in Japanese subjects, showed that the risk of pancreatic cancer was higher among those with the non-O blood type than those with the O blood type[14]. The distribution of the ABO blood type in our overall pancreatic cancer patients was similar to that reported in the Nakao's article. In fact, when the ABO blood type distribution in our pancreatic cancer patients was compared with their cases[14], univariate analysis with the chi-square test showed no difference between them ($P = 0.56$). Moreover, considering that the frequency of the O blood type in our patients was lower than that observed in the general Japanese population, our study provided indirect evidence that the O blood type may be associated with a lower risk of pancreatic cancer in Japanese people.

Another interesting finding is that the B blood type is more common in pancreatic cancer patients with long-standing type 2 diabetes than in those without diabetes. The association between the ABO blood type and diabetes is controversial. Advances in genome-wide sequencing have provided novel insights into the pathogenesis of diabetes mellitus. A recent GWAS showed that genetic variants in the ABO locus were associated with not only diabetes risk, with blood group B showing a decreased risk compared with blood group O[19], but also the plasma levels of soluble intercellular adhesion molecule 1 and soluble E selectin[19-22], both of which are markers of inflammation and are thought to be related to the risk of type 2 diabetes mellitus[23,24]. In addition, a SNP at the ABO locus was reported to be strongly associated with serum tumor necrosis factor alpha[25], which is a pro-inflammatory cytokine that modulates rates of pancreatic ductal cell apoptosis[26], and an adipocytokine that has been implicated in the development of insulin resistance[27]. Although the mechanism underlying the association between ABO blood type, diabetes and pancreatic cancer has not been clarified, these findings suggest interactions among ABO blood types, inflammatory markers, type 2 diabetes and pancreatic cancer.

A major strength of this study is a large cohort of pancreatic cancer patients. Our study has several limitations. First, the major limitation is the lack of an appropriate control group comprising long-standing type 2 diabetes patients without pancreatic cancer. Although our finding showed that the B blood type is more common among pancreatic cancer patients with long-standing type 2 diabetes, a prospective cohort study of diabetics is warranted to confirm whether long-term diabetics with the B blood type have an increased risk of pancreatic cancer. Second, because the study subjects were selected from one hospital, the generalization of our results to other populations is unclear. As mentioned above, with regard to the distribution of the ABO blood type, pancreatic cancer cases in Nakao's study were comparable to ours. Thus, our subjects are not particularly unique. Third, the history of diabetes was mainly based on self-reporting, and the accuracy of the self-reported information is unknown. However, because it is unlikely that a patient would be forgetful regarding the minimum duration of 3 years, a self-report that the duration was 3 years or more than 3 years was likely to be reliable. Fourth, we cannot exclude the possibility that the significant differences observed in ABO blood types among the 3 subgroups were due to chance because of the small number of subjects in each subgroup. This issue warrants further examination in a larger population.

In summary, the retrospective examination of a large cohort of pancreatic cancer patients showed that the B blood type is more common in pancreatic cancer patients with long-standing type 2 diabetes than in those without diabetes. Further studies are needed to better define the set of factors associated with an increased susceptibility to pancreatic cancer in diabetic patients.

COMMENTS

Background

Pancreatic cancer is a dismal disease and refractory to almost all current therapies. Because of the poor prognosis, identifying high-risk individuals and modifying risk factors are important strategies for preventing pancreatic cancer. Currently, smoking habits and type 2 diabetes are well-known modifiable risk factors for pancreatic cancer. However, the prevalence of smoking and the increasing incidence of type 2 diabetes in the general population do not justify screening all subjects for the early detection of pancreatic cancer.

Research frontiers

Recently, there has been emerging evidence that the ABO blood type is associ-

ated with pancreatic cancer risk. A prospective cohort study noted an elevated risk of incidental pancreatic cancer among subjects with blood type A, AB or B compared with blood type O, and those with blood type B had the highest risk.

Innovations and breakthroughs

In their retrospective examination of a large cohort of pancreatic cancer patients, authors found that the distribution of the ABO blood type seemed to differ between patients with long-standing type 2 diabetes and those without, with the former showing a higher frequency of blood type B. In addition, when they divided the former group into 3 subgroups according to the duration of diabetes, they found that there may be several subgroups associated with a specific blood type and characterized by the duration of diabetes. These findings suggest that long-standing type 2 diabetes and other underlying factors, such as blood type and period of diabetes, may play a role in predisposing diabetic patients to pancreatic cancer.

Applications

Although their results should be replicated in prospective studies, they may be useful to define a subset of diabetics that is associated with increased susceptibility to pancreatic cancer.

Terminology

Long-standing type 2 diabetes: Type 2 diabetes represents a complex interaction between hereditary conditions and environmental factors and is essentially different from diabetes secondary to pancreatic cancer. Long-standing diabetes here is defined as diabetes for at least 3 years prior to the diagnosis of pancreatic cancer. This duration should be sufficient to rule out diabetes secondary to the tumor due to the rapid fatal course of pancreatic cancer.

Peer review

Nice retrospective study that is well supported by advanced statistical methodology. Extremely well written in idiomatic English, and limitations are appropriately described. All in all, a good study with marginal clinical relevance.

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BRIEF ARTICLE

Computed tomography findings for predicting severe acute hepatitis with prolonged cholestasis

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Abstract

AIM: To evaluate the significance of computed tomography (CT) findings in relation to liver chemistry and the clinical course of acute hepatitis.

METHODS: Four hundred and twelve patients with acute hepatitis who underwent enhanced CT scanning were enrolled retrospectively. Imaging findings were analyzed for the following variables: gallbladder wall thickness (GWT), arterial heterogeneity, periportal tracking, number and maximum size of lymph nodes, presence of ascites, and size of spleen. The serum levels of alanine aminotransferase, alkaline phosphatase, bilirubin, albumin, and prothrombin time were measured on the day of admission and CT scan, and laboratory data were evaluated every 2-4 d for all subjects during hospitalization.

RESULTS: The mean age of patients was 34.4 years, and the most common cause of hepatitis was hepatitis A virus (77.4%). The mean GWT was 5.2 mm. The number of patients who had findings of arterial heterogeneity, periportal tracking, lymph node enlargement > 7 mm, and ascites was 294 (80.1%), 348 (84.7%), 346 (84.5%), and 56 (13.6%), respectively. On multivariate logistic regression, male gender [odds ratio (OR) = 2.569, 95%CI: 1.477-4.469, P = 0.001], toxic hepatitis (OR = 3.531, 95%CI: 1.444-8.635, P = 0.006), level of albumin (OR = 2.154, 95%CI: 1.279-3.629, P = 0.004), and GWT (OR = 1.061, 95%CI: 1.015-1.110, P = 0.009) were independent predictive factors for severe hepatitis. The level of bilirubin (OR = 1.628, 95%CI: 1.331-1.991, P < 0.001) and GWT (OR = 1.172, 95%CI: 1.024-1.342, P = 0.021) were independent factors for prolonged cholestasis in multivariate analysis.

CONCLUSION: In patients with acute hepatitis, GWT on CT scan was an independent predictor of severe hepatitis and prolonged cholestasis.

Key words: Acute hepatitis; Cholestasis; Computed tomography; Prognosis; Gallbladder

Core tip: Previous studies on the correlation between imaging and laboratory findings in acute hepatitis have shown conflicting results. This study revealed a correlation between abdominal computed tomography findings and liver biochemical parameters. In particular, gallbladder wall thickness (GWT) was the only independent imaging finding that predicts severe hepatitis and prolonged cholestasis. The results of this study suggest that GWT measurement, which is a relatively easy and objective procedure, is helpful to predict severe acute hepatitis or prolonged cholestasis.

Park SJ, Kim JD, Seo YS, Park BJ, Kim MJ, Um SH, Kim CH, Yim HJ, Baik SK, Jung JY, Keum B, Jeen YT, Lee HS, Chun HJ, Kim CD, Ryu HS. Computed tomography findings for predicting severe acute hepatitis with prolonged cholestasis. *World J Gastroenterol* 2013; 19(16): 2543-2549 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i16/2543.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i16.2543

INTRODUCTION

The principal causes of acute hepatitis include viral infection, use of certain drugs, heavy alcohol consumption, chemicals and autoimmunity. In laboratory testing, an increase in serum aminotransferase provides a basis for diagnosis; however, other tests may be conducted to determine the cause of disease and assess severity and prognosis. Abdominal ultrasonography and computed tomography (CT) may help to exclude other conditions that resemble acute hepatitis, such as biliary obstruction, cirrhosis, malignant metastasis to the liver, and diseases that alter liver biochemistry[1].

The general findings of abdominal ultrasonography in acute hepatitis include hepatomegaly, increased periportal echogenicity, decreased echogenicity in liver parenchyma and thickening of the gallbladder wall[2]. Computed tomography may also reveal lymphadenopathy around the hepatoduodenal ligament, fatty deposits around the liver, gallbladder changes, periportal tracking, hepatomegaly, splenomegaly and fluid retention within the pelvis in patients with acute hepatitis A[3].

The purpose of this study was to evaluate the significance of CT findings in relation to liver chemistry and the clinical course in patients with acute hepatitis.

MATERIALS AND METHODS

Patients

We conducted this study through a retrospective review of the medical records of 435 patients who had been hospitalized with acute hepatitis and examined with CT from January 2006 to May 2010. Patients who had previous chronic diseases, such as congestive heart failure, pulmonary diseases, chronic renal insufficiency, or uncontrolled diabetes mellitus, were not included. Twenty-three patients were also excluded because of a lack of relevant clinical chemistry findings or CT imaging obtained without contrast medium. The patients were treated with general symptomatic care with proper hydration and hepatotonic. The clinical and CT data of the 412 eligible patients were analyzed for this study. The cause of acute hepatitis was determined through obtaining a thorough medical history of alcohol consumption, drug use, and coexisting diseases, performing various serological and polymerase chain reaction (PCR) assays to diagnose a variety of viral, bacterial and protozoal infections of the liver caused by hepatitis viruses, cytomegalovirus, Epstein-Barr virus, human immunodeficiency virus, *Toxoplasma*, *Leptospira*, *Candida*, *Mycobacteria*, *Brucella*, pneumocystis, and if necessary, special biochemical tests for metabolic or hereditary hepatic diseases, including serum ceruloplasmin and 24-h urine copper quantification for Wilson's disease. The institutional review board approved this study and waived the written informed consent requirement.

Laboratory examinations

The serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, albumin and prothrombin time (PT) were determined with a conventional autoanalyzer using commercial reagents on the day of admission and CT scan. These same examinations were repeated every 2-4 d for all subjects during hospitalization.

Diagnosis and definitions

The diagnosis of viral hepatitis was based on the positivity of hepatitis A virus, hepatitis B virus, or hepatitis C virus (HCV) markers. The laboratory criteria for confirming each type of acute hepatitis were as follows: acute hepatitis A, positive immunoglobulin M (IgM) antibody to hepatitis A virus; and acute hepatitis B, positive IgM antibody to hepatitis B core antigen and positive hepatitis B surface antigen with seroconversion at least 6 mo after initial presentation. Acute hepatitis C was considered to be present if the following criteria were met: an elevated serum ALT level with a documented normal level during the year before admission, no previous medical history of chronic hepatitis C, positive HCV RNA by PCR with known or suspected exposure to HCV within the preceding four months, and seroconversion of anti-HCV antibody[4,5]. Autoimmune hepatitis was diagnosed based on the recommendations of the International Autoimmune Hepatitis Group[6]. Toxic hepatitis (drug-induced liver injury) was confirmed in patients who had taken relevant various causative medications, herbs, or other xenobiotics within two months before admission and the aforementioned viral markers were all negative.

To identify a relationship between the severity of acute hepatitis and CT findings, we divided patients into two groups, one with and one without severe hepatitis (defined as serum bilirubin $\geq$ 10 mg/dL or PT $\leq$ 40% despite the administration of vitamin K in the most severe phase)[7,8]. To determine the factors associated with prolonged cholestasis, we arbitrarily divided patients into those with serum bilirubin $\geq$ 10 mg/dL for longer than

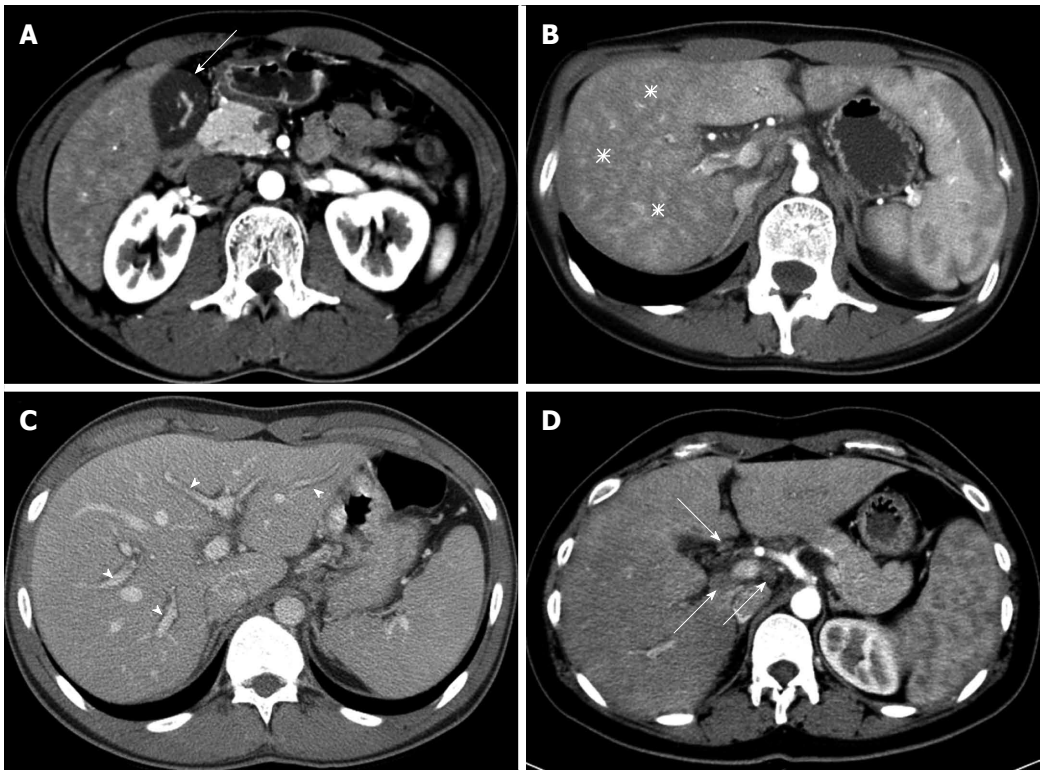

Figure 1 Typical multi-channel computed tomography findings in patients with acute hepatitis. A: Gallbladder wall thickening (arrow); B: Arterial heterogeneity (asterisks), diffuse heterogeneous attenuation of liver parenchyma in the arterial phase; C: Periportal tracking (arrowheads), decreased attenuation, which highlights the portal vein; D: Lymphadenopathy (arrows) in portal hepatis. Other usual findings, *e.g.*, ascites and splenomegaly, are not shown in this figure.

14 d and those having lower bilirubin levels or higher levels for less than 14 d.

Imaging

All examinations were performed with a 64-channel CT scanner (Brilliance 64, Phillips Medical Systems, Cleveland, OH, United States). The scanning parameters used were as follows: tube voltage, 120 kV; effective tube current, 200 mAs with dose modulation (D-Dom, Phillips Medical System); rotation time, 0.5 s; and collimation, 64 mm × 0.625 mm. In all patients, the abdominal CT was conducted with contrast enhancement. The delay between contrast medium administration and the commencement of scanning was determined individually for each patient using standard bolus-tracking software (Automatic Bolus Tracking, Phillips Medical Systems). Scanning began 7 s after a threshold attenuation of 300 HU was reached in the suprarenal aorta. For each patient, 100 mL of iomeprol (400 mg iodine per mL, Iomeron 400; Bracco, Milan, Italy) was injected into an antecubital vein. Contrast medium was injected monophasically at a rate of 3 mL/s. The portal venous and delayed phases started 75 and 120 s later, respectively.

Abdominal CT analysis

Two radiologists (Park BJ and Kim MJ, each with 10 years of experience in abdominal imaging) interpreted the CT images and reached an opinion by consensus. Both radiologists were blinded to the patient's condition and blood test results. Six features of the CT image were interpreted (Figure 1): (1) gallbladder wall thickness (GWT) where the gallbladder is vertical to the surface of the liver in the part adjacent to the liver; (2) non-homogeneity of liver parenchyma in the arterial phase (arterial heterogeneity); (3) decreased attenuation along the portal vein, which is usually expressed as periportal tracking; (4) the maximum size and number of lymph nodes around the liver; (5) presence of ascites; and (6) size of the spleen.

Statistical analysis

The results were presented as the mean ± SD or number of patients (%) as appropriate. The Mann-Whitney *U* test was used to compare groups of continuous data. Categorical data were evaluated using Fisher's exact test or the χ^2 test. Correlations between two continuous variables were assessed using linear regression analysis with the Spearman correlation coefficient. Multivariate analysis was conducted on variables with $P < 0.1$ in univariate analysis using logistic regression analysis to identify factors related to the severity of acute hepatitis or prolonged cholestasis. *P* values less than 0.05 were considered statistically significant. SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis.

RESULTS

Baseline characteristics of the patients

The mean age (± SD) of 412 patients at the time of diagnosis was 34.4 (± 11.4) years (range, 11-92 years),

Table 1 Baseline characteristics of the patients *n* (%)

Characteristics	Total (*n* = 412)	Severe hepatitis (*n* = 92)	Non-severe hepatitis (*n* = 320)	*P* value	Prolonged cholestasis (*n* = 21)	Non-prolonged cholestasis (*n* = 391)	*P* value
Age[1] (yr)	34.4 ± 11.4	36.5 ± 11.5	33.8 ± 11.4	0.035	42.6 ± 15.0	34.0 ± 11.1	0.013
Gender, male	237 (57.5)	62 (67.4)	175 (54.7)	0.032	11 (52.4)	226 (57.8)	0.655
Etiology							
Hepatitis A	319 (77.4)	67 (72.8)	252 (78.8)		8 (38.1)	311 (79.5)	
Toxic	44 (10.7)	17 (18.5)	27 (8.4)		10 (47.6)	34 (8.7)	
Unknown	25 (6.1)	1 (1.1)	24 (7.5)		0 (0)	25 (6.4)	
Hepatitis B	16 (3.9)	4 (4.3)	12 (3.8)		2 (9.5)	14 (3.6)	
Alcohol	4 (1.0)	1 (1.1)	3 (0.9)		0 (0)	4 (1.0)	
Hepatitis C	2 (0.5)	1 (1.1)	1 (0.3)		1 (4.8)	1 (0.3)	
Autoimmune	2 (0.5)	1 (1.1)	1 (0.3)		0 (0)	2 (0.5)	
Etiology, toxic	44 (10.7)	17 (18.5)	27 (8.4)	0.001	10 (47.6)	34 (8.7)	< 0.001
ALT[1] (IU/L)	2053 ± 1759	2145 ± 2207	2027 ± 1611	0.392	998 ± 1090	2110 ± 1772	0.001
ALP[1] (IU/mL)	155 ± 75	152 ± 72	156 ± 76	0.983	153 ± 110	156 ± 73	0.265
Bilirubin[1] (mg/dL)	5.6 ± 4.9	11.2 ± 6.7	3.9 ± 2.6	< 0.001	18.6 ± 6.9	4.9 ± 3.7	< 0.001
Albumin[1] (g/dL)	3.9 ± 0.4	3.8 ± 0.4	4.0 ± 0.4	< 0.001	3.8 ± 0.4	3.9 ± 0.4	0.174
PT[1] (%)	81.0 ± 18.0	75.2 ± 22.7	83.7 ± 16.6	0.003	84.6 ± 15.0	81.7 ± 18.6	0.721
GWT[1], mm	5.2 ± 5.5	7.2 ± 6.0	5.5 ± 5.3	0.009	9.6 ± 6.7	5.7 ± 5.4	0.003
> 3	233 (56.5)	70 (70.7)	168 (51.7)	0.004	17 (81.0)	216 (55.2)	0.025
> 7	142 (34.5)	36 (39.1)	106 (33.1)	0.040	11 (52.4)	131 (33.5)	0.005
Arterial heterogeneity (+)	294 (80.1)	68 (73.9)	226 (70.6)	0.755	15 (71.5)	279 (71.3)	1.000
Periportal tracking (+)	348 (84.7)	78 (84.8)	270 (84.4)	0.869	18 (85.7)	330 (84.4)	1.000
LN number[1]	4.8 ± 3.0	5.1 ± 3.3	4.8 ± 2.9	0.581	5.3 ± 3.7	4.8 ± 3.0	0.701
LN size[1] (cm)	0.97 ± 0.26	0.99 ± 0.28	0.96 ± 0.26	0.465	0.97 ± 0.30	0.97 ± 0.26	0.743
⩾ 0.8	346 (84.5)	79 (85.9)	267 (83.4)	0.632	17 (81.0)	329 (84.1)	0.759
> 1	162 (39.3)	42 (45.7)	120 (37.5)	0.183	10 (47.6)	152 (38.9)	0.494
Ascites (+)	56 (13.6)	17 (18.5)	39 (12.2)	0.123	7 (33.3)	49 (12.5)	0.015
Spleen size[1] (cm)	10.9 ± 1.6	11.04 ± 1.68	10.84 ± 1.60	0.419	10.44 ± 1.7	10.91 ± 1.6	0.094
⩾ 12	95 (23.1)	24 (26.1)	71 (22.2)	0.483	3 (14.3)	92 (23.5)	0.431

[1]Data presented as mean ± SD. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; PT: Prothrombin time; GWT: Gallbladder wall thickness; LN: Lymph node.

and 237 (57.5%) were male. The most common cause of acute hepatitis was hepatitis A virus (n = 319, 77.4%). The other causes in order of frequency were toxicity, indeterminate, hepatitis B virus, alcoholism, HCV, and autoimmunity. Contrast-enhanced abdominal CT was performed at 1.5 d, on average, after admission (the second hospitalization day). The mean GWT on CT was 5.2 (± 5.5) mm, and 233 patients (56.5%) showed thickening of over 3 mm. The numbers of patients who showed arterial heterogeneity, periportal tracking, enlarged lymph nodes greater than 7 mm, and presence of ascites were 294 (80.1%), 348 (84.7%), 346 (84.5%), and 56 (13.6%), respectively. Spleen enlargement by greater than 12 cm was found in 95 patients (23.1%) (Table 1).

Correlation between CT findings and liver chemistry

GWT was related to serum ALT (r = 0.234, P < 0.001), ALP (r = 0.180, P < 0.001), bilirubin (r = 0.319, P < 0.001), albumin (r = -0.282, P < 0.001), and PT (r = -0.118, P = 0.017). Lymph node size was correlated with serum ALT (r = 0.164, P = 0.001), ALP (r = 0.135, P = 0.006), and bilirubin (r = 0.138, P = 0.005). The number of lymph nodes was correlated with serum ALP (r = 0.150, P = 0.003) and bilirubin (r = 0.202, P < 0.001). The spleen size was correlated with serum ALT (r = 0.119, P = 0.015) and bilirubin (r = 0.190, P < 0.001). Serum ALT levels (P < 0.001) and PT (P = 0.032) varied depending on the presence of arterial heterogeneity. The presence of periportal tracking was related to serum ALP (P = 0.007), bilirubin (P < 0.001), and albumin (P = 0.002). The presence of ascites was related to serum ALT (P = 0.005), bilirubin (P = 0.012), and albumin (P < 0.001) levels and PT (P = 0.006) (Table 2).

Factors associated with acute hepatitis severity

On univariate analysis, age, gender, frequency of toxic hepatitis, serum bilirubin and albumin, PT at the time of CT scanning and GWT showed significant differences (P < 0.05) between the group with severe hepatitis (defined as bilirubin ⩾ 10 mg/dL or PT ⩽ 40% in the most severe phase) and the group with non-severe hepatitis. On multivariate analysis, male gender [odds ratio (OR) = 2.569, 95%CI: 1.477-4.469, P = 0.001], toxic hepatitis (OR = 3.531, 95%CI: 1.444-8.635, P = 0.006), low serum albumin (OR = 2.154, 95%CI: 1.279-3.629, P = 0.004), and high GWT (OR = 1.061, 95%CI: 1.015-1.110, P = 0.009) independently predicted severe hepatitis (Tables 1 and 3).

Factors associated with prolonged cholestasis

Patient age, frequency of toxic hepatitis, serum ALT and bilirubin levels at the time of CT scan, GWT, and the frequency of ascites differed significantly between patients with prolonged cholestasis and those with non-prolonged

Table 2 Relationship between computed tomography findings and liver chemistry (mean ± SD)

	GWT	LN maximum size	LN number	Spleen size	Arterial heterogeneity			Periportal tracking			Ascites		
					Absence (n = 73)	Presence (n = 294)	P value	Absence (n = 63)	Presence (n = 348)	P value	Absence (n = 356)	Presence (n = 56)	P value
ALT (IU/L)	0.234 (< 0.001)	0.164 (0.001)	-0.013 (0.798)	0.119 (0.015)	1504 ± 1478	2216 ± 1794	< 0.001	1994 ± 2139	2064 ± 1688	0.074	1981 ± 1769	2509 ± 1641	0.005
ALP (IU/mL)	0.180 (< 0.001)	0.135 (0.006)	0.150 (0.003)	0.086 (0.082)	145 ± 76	159 ± 75	0.108	133 ± 63	159 ± 76	0.007	154 ± 76	164 ± 66	0.152
Bilirubin (mg/dL)	0.319 (< 0.001)	0.138 (0.005)	0.202 (< 0.001)	0.190 (< 0.001)	5.6 ± 5.2	5.6 ± 4.9	0.451	4.3 ± 5.1	5.8 ± 4.9	< 0.001	5.3 ± 4.7	7.0 ± 5.9	0.012
PT (s)	-0.118 (0.017)	-0.038 (0.445)	0.072 (0.149)	-0.092 (0.061)	86 ± 16	81 ± 18	0.032	81 ± 20	82 ± 18	0.795	82 ± 18	76 ± 17	0.006
Albumin (g/dL)	-0.282 (< 0.001)	-0.018 (0.717)	-0.017 (0.739)	-0.062 (0.206)	4.0 ± 0.4	3.9 ± 0.4	0.404	4.1 ± 0.4	3.9 ± 0.4	0.002	4.0 ± 0.4	3.7 ± 0.4	< 0.001

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; PT: Prothrombin time; GWT: Gallbladder wall thickness; LN: Lymph node.

Table 3 Multivariate logistic regression analysis for the factors associated with severe acute hepatitis and prolonged cholestasis in acute hepatitis

Variables	OR	95%CI for OR		P value
		Lower	Upper	
Severe acute hepatitis				
Age (yr)	1.007	0.928	1.033	0.579
Gender, male	2.569	1.477	4.469	0.001
Etiology, toxic	3.531	1.444	8.635	0.006
Albumin (g/dL)	2.154	1.279	3.629	0.004
GWT (mm)	1.061	1.015	1.110	0.009
Prolonged cholestasis in acute hepatitis				
Age (yr)	0.393	0.059	2.599	0.333
Etiology, toxic	6.848	0.960	48.859	0.055
ALT (IU/L)	1.000	1.000	1.001	0.607
Bilirubin (mg/dL)	1.628	1.331	1.991	< 0.001
GWT (mm)	1.172	1.024	1.342	0.021
Ascites	0.484	0.119	6.039	0.869

GWT: Gallbladder wall thickness; ALT: Alanine aminotransferase; OR: Odds ratio.

cholestasis (Table 1). In this study, prolonged cholestasis was defined as serum bilirubin levels of 10 mg/dL or higher sustained for longer than 14 d. On multivariate analysis, elevated serum bilirubin (OR = 1.628, 95%CI: 1.331-1.991, $P < 0.001$) and increased GWT (OR = 1.172, 95%CI: 1.024-1.342, $P = 0.021$) were independently predictive for prolonged cholestasis. Three hundred and fifty-six of 357 patients with bilirubin levels less than 10 mg/dL at the time of CT scan were assigned to the non-prolonged cholestasis group. Among patients with serum bilirubin levels ⩾ 10 mg/dL (n = 55), 79% (22/28) of patients with GWT less than 5 mm were classified in the non-prolonged cholestasis group, and 71% (5/7) of those with GWT of 13 mm or higher were classified in the prolonged cholestasis group (Tables 1 and 3).

DISCUSSION

Hepatomegaly, increased periportal echogenicity, decreased parenchymal echogenicity, lymph node enlargement, and a thickened gallbladder wall are frequently observed during the ultrasonographic examination of patients with acute hepatitis[2,9-12]. Similar findings also appear on CT scan[13-16], and CT easily detects lymphadenopathy and gallbladder wall thickening. The "periportal tracking" observed on CT may correspond to the same pathology as "increased periportal echogenicity" on ultrasonography. The non-homogeneity of liver parenchyma observed in the arterial phase of dynamic CT (arterial heterogeneity) is a CT-specific image generated by contrast media in patients with acute hepatitis[17]. These image findings are most commonly observed at the icteric phase of acute hepatitis[3].

Thickening of the gallbladder wall has attracted attention because it may be detected by ultrasonography in 51%-91% of patients with acute hepatitis[18-22]. In a recent study, ultrasonography within 7 d after symptomatic onset of acute hepatitis showed morphological changes in the gallbladder in more than 80% of patients, and these findings normalized as the hepatitis improved clinically[20]. In the present study, 56.6% of patients with acute hepatitis demonstrated an abnormal GWT when GWT > 3 mm was defined as abnormal. In addition, we observed other CT findings in patients with acute hepatitis, including lymph node enlargement, periportal tracking, and arterial heterogeneity at frequencies of 84.5%, 84.7% and 80.1%, respectively. These values were all higher than the frequency of GWT.

Correlations between imaging and laboratory findings in acute hepatitis have not been closely investigated. Studies on the correlation between GWT and the liver chemistry index show conflicting results. Some studies show that GWT increases at elevated serum aminotransferase levels[22,23], while other studies do not show this correlation[18,19,24]. Suk *et al*[24] showed a significant correlation between GWT and increases in serum bilirubin. In the present study, GWT clearly correlated with elevated serum bilirubin and liver enzymes, which supports the hypothesis that inflammation and necrosis in the liver parenchyma induce an inflammatory reaction and hyperemia in muscular and serosal layers of the gallbladder

wall[23]. We also found a negative correlation between GWT and serum albumin levels, which suggests that hypoalbuminemia caused by acute hepatitis contributes to gallbladder wall edema. Patients with chronic renal insufficiency or cirrhosis who also have hypoalbuminemia frequently present gallbladder wall thickening[14].

In the present study, imaging findings other than GWT showed significant correlations with the serum levels of ALT, ALP, bilirubin, albumin and PT. These imaging findings included lymph node enlargement around the hepatoduodenal ligament, arterial heterogeneity, and periportal tracking. These correlations suggested that image findings may reflect liver damage and hepatic dysfunction, at least to some degree (Table 2). We therefore tested the prognostic value of imaging data in patients with acute hepatitis. First, we designated patients with serum bilirubin levels ≥ 10 mg/dL or PT index ≤ 40% despite the administration of vitamin K as having severe hepatitis, and we then sought to identify factors that may predict this outcome. In our analysis, male gender, toxic hepatitis, low serum albumin, and increased GWT independently predicted severe hepatitis. Furthermore, increased GWT and high serum bilirubin levels at the time of CT scanning independently predicted prolonged cholestasis. Because only approximately 20% of patients with hyperbilirubinemia (serum bilirubin > 10 mg/dL) at the time of CT scan showed prolonged cholestasis if their GWT was < 5 mm, the detection of GWT may be of value in predicting prolonged cholestasis in patients with acute hepatitis. Of course, other imaging modalities could substitute for CT scan unless CT is absolutely necessary, especially in young patients, to avoid unnecessary radiation exposure if they could also depict changes in the gallbladder, considering arguments on the safety of radiation exposure caused by CT scan in young patients[25,26].

This study is limited by its retrospective design. Therefore, we could not investigate the correlation between imaging findings and the duration of hospitalization because the criteria for patient discharge were not set in advance. Similarly, we could not determine the time of symptomatic onset of hepatitis in many patients. However, this study is strengthened by its size, which allowed us to confirm significant correlations between imaging findings and liver chemistry data in patients with acute hepatitis. In addition, this study revealed the value of GWT for predicting the outcome of acute hepatitis.

In conclusion, abdominal CT findings and liver biochemical parameters in patients with acute hepatitis showed some correlations, and GWT was the only imaging variable that independently predicted the severity of acute hepatitis or prolonged cholestasis.

COMMENTS

Background

In patients with suspected acute hepatitis, imaging studies, such as ultrasonography or computed tomography (CT), are usually performed to rule out other diseases presenting with similar clinical and biochemical abnormalities (*e.g.*, extrahepatic cholestasis, diffuse metastatic disease or cirrhosis).

Research frontiers

Studies on the correlation between imaging and laboratory findings in acute hepatitis have shown conflicting results.

Innovations and breakthroughs

This large-scale study has revealed significant correlations between abdominal CT findings and liver biochemical parameters. Furthermore, gallbladder wall thickness (GWT), which can be easily measured by other imaging techniques as well, was found to be valuable for predicting the outcome of patients with acute hepatitis.

Applications

The results of this study suggest that GWT measurement by imaging modalities is helpful to classify patients with severe acute hepatitis or prolonged cholestasis.

Terminology

GWT refers to the thickness of the gallbladder wall vertical to the surface of the liver in the part adjacent to the liver.

Peer review

The authors describe the CT findings of severe acute hepatitis in 412 hospitalized patients. They found that GWT was an independent predictive factor for severe hepatitis. My main criticism is the relevance of such a paper. This study involved exposing young people to unnecessary radiation.

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BRIEF ARTICLE

New-style laparoscopic and endoscopic cooperative surgery for gastric stromal tumors

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Abstract

AIM: To evaluate the feasibility and safety of a new style of laparoscopic and endoscopic cooperative surgery (LECS), an improved method of laparoscopic intragastric surgery (LIGS) for the treatment of gastric stromal tumors (GSTs).

METHODS: Six patients were treated with the new-style LECS. Surgery was performed according to the following procedures: (1) Exposing and confirming the location of the tumor with gastroscopy; (2) A laparoscopy light was placed in the cavity using the trocar at the navel, and the other two trocars penetrated both the abdominal and stomach walls; (3) With gastroscopy monitoring, the operation was carried out in the gastric lumen using laparoscopic instruments and the tumor was resected; and (4) The tumor tissue was removed orally using a gastroscopy basket, and puncture holes and perforations were sutured using titanium clips.

RESULTS: Tumor size ranged from 2.0 to 4.5 cm (average 3.50 ± 0.84 cm). The operative time ranged from 60 to 130 min (average 83.33 ± 26.58 min). Blood loss was less than 20 mL and hospital stay ranged from 6 to 8 d (average 6.67 ± 0.82 d). The patients were allowed out of bed 12 h later. A stomach tube was inserted for 72 h after surgery, and a liquid diet was then taken. All cases had single tumors which were completely resected using the new-style LECS. No postoperative complications occurred. Pathology of all resected specimens showed GST: no cases of implantation or metastasis were found.

CONCLUSION: New-style LECS for GSTs is a quick, optimized, fast recovery, safe and effective therapy.

Key words: Laparoscopic and endoscopic cooperative surgery; Gastric stromal tumor

Core tip: A new style of laparoscopic and endoscopic cooperative surgery (LECS) was used to treat gastric stromal tumors (GSTs) originating from the muscularis propria in this study. The operation was carried out in the gastric cavity, and the GST was completely removed using a gastroscopic light source and laparoscopic instruments. This method is minimally invasive and avoids many complications such as bleeding and intra-abdominal infection. Furthermore, the integrity of stomach structure and function is preserved. New-style LECS is a safe and effective method for the treatment of GSTs.

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INTRODUCTION

Gastric stromal tumors (GSTs) are potentially malignant tumors, accounting for approximately 60%-70% of gastrointestinal stromal tumors (GISTs)[1]. GSTs diffuse mainly by hematogenous metastasis and direct violation, are less likely to occur in lymph metastasis, and are not sensitive to chemotherapy or radiotherapy. The main treatment option is complete tumor resection[2-5]. Laparoscopic and endoscopic cooperative surgery (LECS) is an important therapy in the treatment of GSTs[6-8]. In this study, we used this new improved method of laparoscopic intragastric surgery (LIGS) to completely resect GSTs, and achieved good results.

MATERIALS AND METHODS

General information

From January 2011 to May 2012, 6 cases of GSTs originating from the muscularis propria were confirmed by endoscopic ultrasound (EUS) and gastroscopy. The patients were 2 males and 4 females, aged 42-63 years, with a mean age of 53.83 ± 7.94 years. All cases had a single tumor with an average size of 3.50 ± 0.84 cm. The tumors were located in the fundus fornix in 3 cases, in the posterior wall of the gastric body in 1 case and in the gastric cardia in 2 cases (Table 1).

Table 1 Patient demographics and gastric stromal tumor characteristics

Patient	Sex	Age (yr)	Tumor location	Tumor size (cm)	Operation time (min)	Hospital stay (d)	Complications
1	M	48	Fundus fornix	3.5	80	7	None
2	F	42	Posterior wall of body	4.5	50	7	None
3	F	56	Cardia	2.5	130	7	None
4	F	63	Fundus fornix	2.0	70	5	None
5	F	53	Cardia	3.0	90	7	None
6	M	61	Fundus fornix	3.5	80	7	None

M: Male; F: Female.

Surgical methods

Using tracheal intubation with general anesthesia, the patients were placed in the supine position with their heads slightly to the left. Both the surgeon and the endoscopist stood on the patient's left side. The location of the tumor was determined by the endoscopist (Figure 1A). The surgeon made a curved incision of approximately 0.5 cm at the superior border of the umbilicus, a CO_2 pneumoperitoneum with pressure of 12 mmHg was established, and a 0.5 cm laparoscope was inserted for observation. Two punctures of about 0.5 cm at the left upper quadrant of the abdomen were then established. Two sutures were placed in the avascular area of the anterior wall of the stomach and exported from one of the puncture holes, in order to pull the anterior stomach wall close to the abdominal wall. The stomach was inflated during gastroscopy. Under laparoscopic monitoring, the surgeon inserted 2 ordinary 0.5 cm puncture cannulas into the stomach from the anterior wall, at a distance of more than 3 cm (Figure 1B and C). Under gastroscopic guidance, the surgeon used an ultrasound knife to completely resect the tumors (Figure 1D). Specimens were removed *via* the mouth using grasping forceps (Figure 1E and F). If there was no perforation, the two puncture cannulas in the gastric lumen were pulled out one by one, and the puncture holes were clipped using titanium clips *via* the endoscope. Otherwise, the perforation was clipped from the edge to the center using clips, the puncture cannulas were then pulled out, and the puncture holes closed by clips (Figure 1H and I). If the stomach was repeatedly inflated without leak, the gas was exhausted and a gastric tube was inserted. The pneumoperitoneum was reestablished and the hanging line in the stomach wall was removed. Intra-abdominal exudate and bleeding was fully suctioned. The pneumoperitoneum was removed when the surgeon pulled the puncture cannula out of the abdominal wall and sutured the puncture subcutaneously.

RESULTS

The average operative time was 83.33 ± 26.58 min and blood loss was less than 20 mL. A stomach tube was inserted for 72 h after surgery, and a liquid diet was then taken. After surgery, there was no significant abdominal pain or pneumoperitoneum. Patients were allowed out of bed 12 h later. The average length of hospital stay was 6.67 ± 0.82 d. Pathological results were GSTs in all patients, and the structure of the basal and cutting edge was normal. On the 3rd and 6th mo after surgery, the patients were reassessed by endoscopy and EUS, and all patients had healed well without metastasis.

DISCUSSION

With the development of endoscopic technology, LECS and endoscopic submucosal dissection (ESD) have been used more and more widely in the treatment of GSTs[6-13]. Conventional LECS, according to the different locations of GSTs, is mainly divided into 2 types: the laparoscope-assisted endoscopic technique (LAET) and endoscope-assisted laparoscopic technique (EALT). In addition, EALT can be divided into endoscope-assisted wedge resection (EAWR) and LIGS[6,8,14,15].

LAET mainly refers to the procedure where laparoscopy is used to closely monitor the endoscopic resection of tumors throughout the surgical process, and the timely treatment of perforation, bleeding and other complications[16]. However, when a tumor is too large or located in the posterior wall or gastric fundus, gastroscopy is very difficult to achieve. In EAWR, the tumor is resected by laparoscopy, while endoscopy plays an important role in the location of tumors, and is usually used for GSTs at the lesser curvature and the anterior wall of the stomach[17]. When the lesions are near the pylorus or cardia, a wedge resection may lead to stenosis[18].

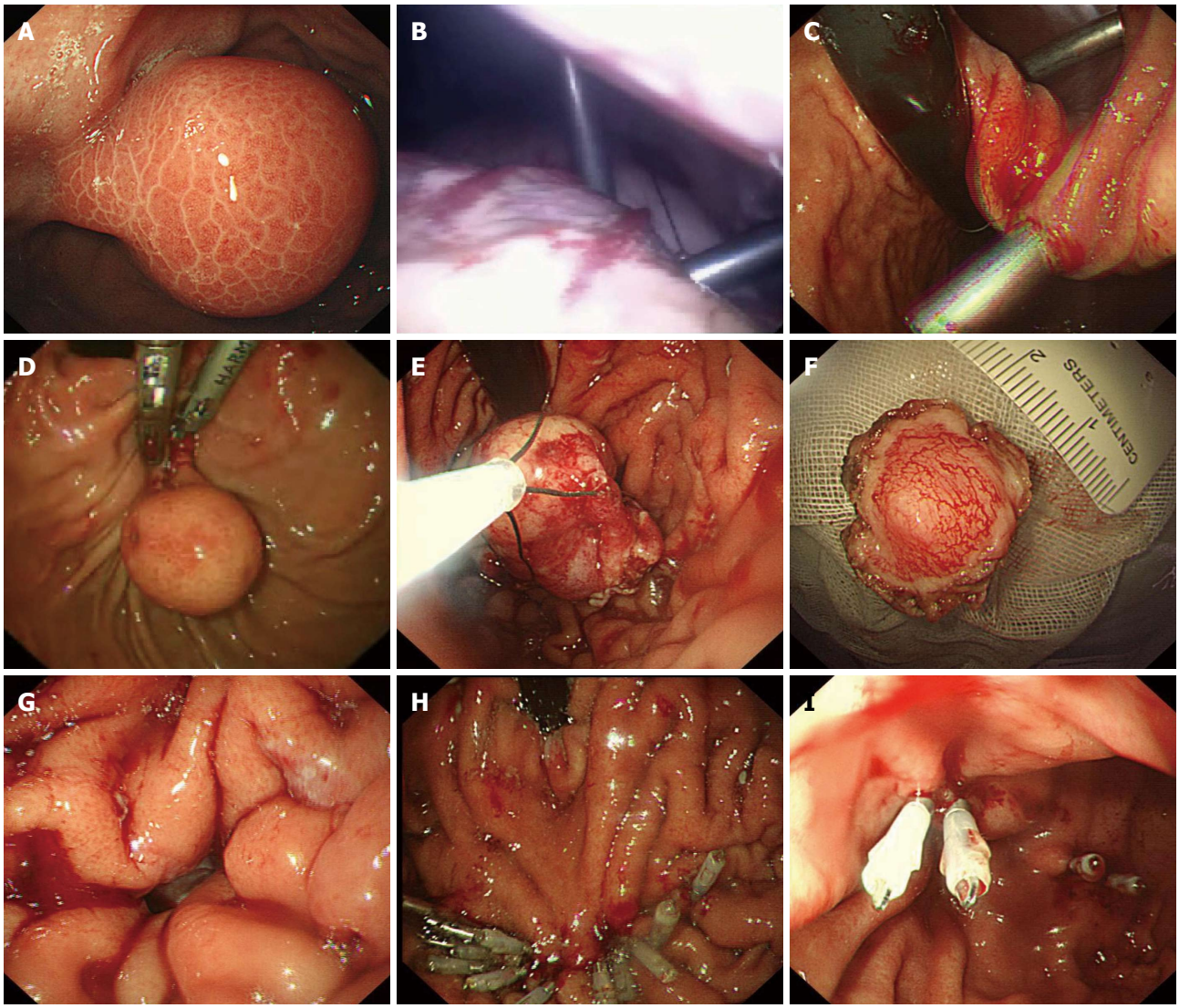

Figure 1 New-style laparoscopic and endoscopic cooperative surgery for gastric stromal tumors. A: Tumor in fundus fornix; B, C: Two puncture cannulas were inserted into the stomach; D: The tumor was resected by ultrasound knife under gastroscopic guidance; E: Specimen was took out *via* the mouth using endoscopic basket; F: The tumor; G: Perforation in fundus. H: Perforation was closed by clips *via* gastroscope; I: The 2 puncture holes in stomach were clipped by clips.

Laparoscopic instruments are used in LIGS, including a laparoscopic light source, to puncture the gastric cavity directly and to carry out the surgery within it. The tumor is then removed from the abdominal wall, and the gastric wall and puncture holes are sutured using laparoscopic instruments[19-21].

Compared with the traditional LIGS, the advantages of the new-type LECS are as follows: (1) Using gastroscopic light, doctors can reduce the laparoscopic light holes in the gastric wall, and close laparoscopic operational holes with clips *via* the gastroscope. Compared with normal laparoscopic suturing, this technique is minimally invasive; (2) Tumors less than 4.5 cm in size can be removed *via* gastroscopy from the mouth to reduce trauma to the abdominal wall; and (3) Both the exterior and interior of the gastric cavity can be observed by laparoscopy and gastroscopy, respectively, in order to reduce the incidence of postoperative complications.

ESD is a technique which uses gastroscopy alone to resect tumors[22-24]. However, for tumors greater than 2 cm or exogenic or mixed type tumors, or if the tumor is located in an area where it is difficult to operate, ESD can result in problems, such as the risk of bleeding or perforation. For large perforations, it is more difficult to suture using clips during gastroscopy. Furthermore, longer operative time, and no peritoneal lavage and suction, can lead to pneumothorax, pneumoperitoneum, abdominal cavity cysts, peritoneal abscesses and other complications[25,26]. Compared to ESD, the advantages of new-type LECS are as follows: (1) Shortens the operative time; (2) Reduces the risk of bleeding; (3) In the event of perforation, the use of laparoscopy to suction exudate and bleeding and to execute peritoneal washing, will reduce the chance of intra-abdominal infection and other complications; and (4) Avoids the situation where observation and the operative field are unclear after perforation.

In conclusion, new-type LECS can be used to resect

GSTs greater than 2 cm in size which originate from the muscularis propria, mixed tumors or where difficulty in resection is encountered by laparoscopy or gastroscopy.

Although this new type of LECS has unique advantages in the treatment of certain GSTs, this surgery has been used for less than one year. It is still at the stage of exploration and practice, and requires more cases to verify its usefulness. In addition, it requires good laparoscopic physicians, endoscopists and endoscopy nurses to help each other to accomplish the safe and effective use of this technique.

COMMENTS

Background

Gastric stromal tumors (GSTs) are potentially malignant tumors, and the main outcome of treatment is complete tumor resection. Laparoscopic and endoscopic cooperative surgery (LECS) is now being used more widely in the treatment of GSTs. In order to decrease complications and minimize trauma, a new type of LECS was used for GSTs in the present study.

Research frontiers

Conventional LECS is used for the treatment of GSTs originating from the muscularis propria. However, use of the new type of LECS has rarely been reported. The authors investigated the clinical safety and efficacy of the new type of LECS for GSTs.

Innovations and breakthroughs

This is the first report on the new type of LECS for GSTs to date. This new surgery is similar to laparoscopic intragastric surgery. The operation is carried out in the stomach and the GST is completely removed using a gastroscopic light source and laparoscopic instruments. The integrity of the stomach structure and function is preserved. It is an innovative and effective operation for GSTs, and has unique advantages in the treatment of certain GSTs.

Applications

A new type of LECS was used for GSTs greater than 2 cm in size which originated from the muscularis propria, in mixed tumors and in tumors which were difficult to resect using only laparoscopy or gastroscopy.

Terminology

LECS is an operation for resecting gastrointestinal tumors using laparoscopy and endoscopy. This method includes a laparoscope-assisted endoscopic technique and an endoscope-assisted laparoscopic technique.

Peer review

The new-type of LECS described in this study is relatively new, and the procedures are feasible and may have some localizing advantages in selected patients.

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BRIEF ARTICLE

Endoscopic mucosal resection for rectal carcinoids under micro-probe ultrasound guidance

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Abstract

AIM: To assess the therapeutic value of endoscopic mucosal resection (EMR) under micro-probe ultrasound guidance for rectal carcinoids less than 1 cm in diameter.

METHODS: Twenty-one patients pathologically diagnosed with rectal carcinoids following colonoscopy in our hospital from January 2007 to November 2012 were included in this study. The patients consisted of 14 men and 7 women, with a mean age of 52.3 ± 12.2 years (range: 36-72 years). The patients with submucosal tumors less than 1 cm in diameter arising from the rectal and muscularis mucosa detected by micro-probe ultrasound were treated with EMR and followed up with conventional endoscopy and micro-probe ultrasound.

RESULTS: All of the 21 tumors were confirmed by micro-probe ultrasound as uniform hypoechoic masses originating from the rectal and muscularis mucosa, without invasion of muscularis propria and vessels, and less than 1 cm in diameter. EMR was successfully completed without bleeding, perforation or other complications. The resected specimens were immunohistochemically confirmed to be carcinoids. Patients were followed up for one to two years, and no tumor recurrence was reported.

CONCLUSION: EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter.

Key words: Micro-probe ultrasound; Endoscopic mucosal resection; Rectal carcinoid; Endoscopic submucosal dissection; Submucosal tumors

Core tip: Rectal carcinoids are rare neuroendocrine tumors that are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage. This study reports on a hospital-based series of 21 patients who were successfully treated and followed up for 1-2 years for small rectal carcinoids less than 1 cm by means of endoscopic mucosal resection (EMR) under micro-probe ultrasound guidance. EMR is found to be a safer option with fewer complications, shorter operation time, and comparable rate of complete removal to endoscopic submucosal dissection, therefore, EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

Zhou FR, Huang LY, Wu CR. Endoscopic mucosal resection for rectal carcinoids under micro-probe ultrasound guidance. *World J Gastroenterol* 2013; 19(16): 2555-2559 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i16/2555.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i16.2555

INTRODUCTION

Rectal carcinoids are rare neuroendocrine tumors that

are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage[1]. With the widespread popularity of colonoscopy, however, the detection rate of rectal carcinoids has been increasing in recent years, enabling us to achieve better outcomes for patients with submucosal tumors (SMTs) derived from the muscular layer of rectal mucosa using endoscopic mucosal resection (EMR) combined with micro-probe ultrasound examination. The following is a summary of a retrospective analysis of 21 patients with pathologically confirmed rectal carcinoid tumors treated in our hospital from January 2007 to November 2012.

MATERIALS AND METHODS

Subjects

Twenty-one patients pathologically diagnosed with rectal carcinoids following colonoscopy in our hospital from January 2007 to November 2012 were included in this study. The patients consisted of 14 men and 7 women, with a mean age of 52.3 ± 12.2 years (range: 36-72 years).

Methods

Equipment: Olympus GIF-260 Endoscope, Fujinon-Sp702 micro-probe system, with a micro-probe frequency of 20 mHz and probe diameter of 2.5 mm.

Procedures: Lesions were assessed by endoscopic ultrasonography (Figures 1-7), and endoscopic submucosal resection was performed for lesions less than 1 cm in diameter and not deeper than the submucosa. Normal saline was injected into the root of each tumor under colonoscopy to form a bulge before EMR. Metal hemoclipping was applied to the excision wounds to prevent postoperative perforation, bleeding and other complications. The removed tissues were sent for pathological identification. All specimens were fixed with 10% formalin, and stained with hematoxylin-eosin for immunohistochemistry in the Department of Pathology.

RESULTS

Colonoscopy was performed for subjects with unexplained changes in bowel habit (including stool frequency and consistency) and those who underwent routine physical examinations, and 21 patients were diagnosed as having rectal carcinoids. Among the 21 cases of rectal carcinoids, 16 (76.2%) cases were located within 8 cm and 5 (23.8%) cases were located within 8-10 cm from the anal margin. The typical submucosal lesions with smooth mucosal surface were the predominant endoscopic manifestations (85.7%, 18/21). Three (14.31%, 3/21) lesions were depressed and erosive at the top region, and 17 (80.9%, 17/21) appeared tough or hard, and only 1 lesion was soft (0.5%). Seventeen (80.9%) cases appeared yellow, and 4 (19.1%) cases appeared red or normal.

All of the 21 tumors were confirmed by micro-probe ultrasound as uniform hypoechoic masses originating from the rectal and muscularis mucosa, without invasion of muscularis propria and vessels, and less than 1 cm in diameter. EMR was successfully completed without bleeding, perforation or other complications. The resected specimens were immunohistochemically diagnosed as carcinoids. The post-operative follow-up lasted 1-2 years, during which colonoscopy was performed in the first month, and then in months 3, 6, 12 and 24. No tumor relapse was noted.

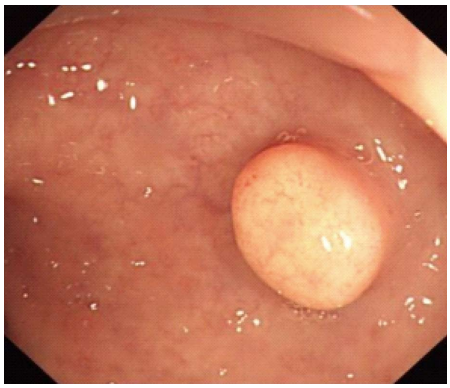

Figure 1 Endoscopic findings of rectal carcinoids. Endoscopy shows yellow or gray submucosal nodules, which are hard and covered by normal-appearing mucosa.

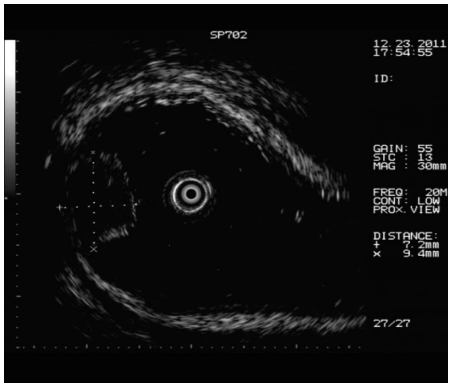

Figure 2 Rectal carcinoids under micro-probe ultrasound. The hypoechoic lesions are mainly derived from submucosa or muscularis mucosa.

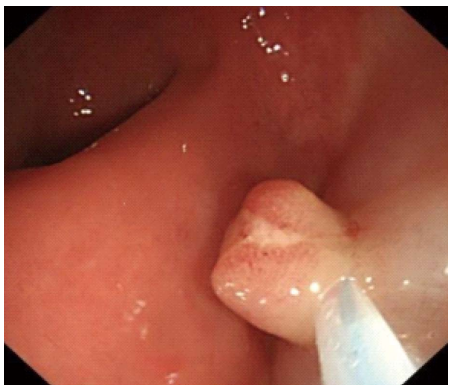

Figure 3 Endoscopic loop ligation after injection.

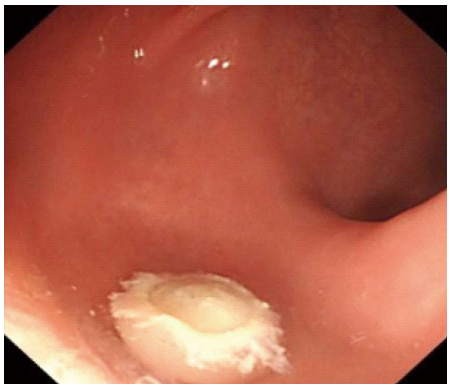

Figure 4 Endoscopic resection of the lesions. The whole rectal carcinoid mass is visible in the endoscopically resected lesion.

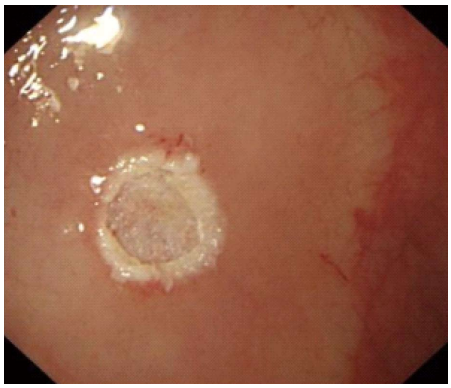

Figure 5 Wounds after endoscopic resection. After the endoscopic resection of the rectal carcinoid, the wound is complete and no residual mass is present.

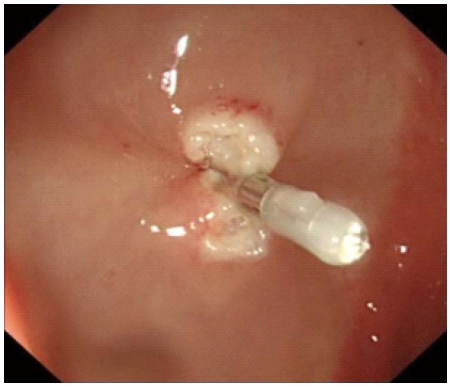

Figure 6 Wounds after endoscopic clipping.

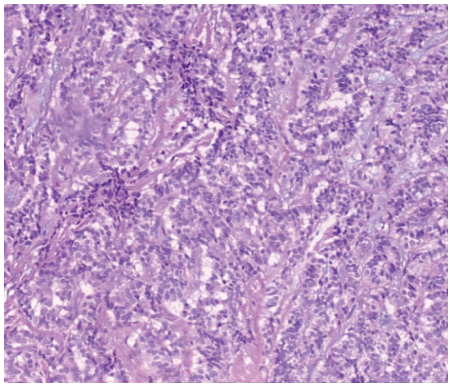

Figure 7 Pathological manifestations of rectal carcinoids. The carcinoid cells are small oval or polygonal, nested or glandular-like, and with a regular and small nucleus under microscope (magnification × 400).

DISCUSSION

Carcinoid tumors are derived from argyrophilic cells in the intestinal glandular base (Kulchitsky cells) and are known for their affinity for silver staining, thus, these tumors are also known as argyrophil cell carcinomas. Rare as they are, carcinoids can occur in multiple body systems, with the gastrointestinal tract being the most commonly involved system. Rectal carcinoids are the third leading gastrointestinal carcinoids, accounting for about 14% of all conditions of this type[1], with an increasing incidence in recent years potentially attributable to extensive endoscopic application and increased awareness of carcinoid tumors. Epidemiological surveys have revealed that the incidence rate of gastrointestinal carcinoids has increased year by year with the popularity of endoscopy, and ranges from 0.27 to 1.33 per 100000 people. The highest incidence was found in the appendix (38%), followed by the small intestine (29%), colon (13%), and stomach (12%). Based on the epidemiological statistics in Europe and North America conducted by Shields *et al*[2], the median age for rectal carcinoids is 55 years, the average tumor size is 10 mm, and the average age of onset for the surveyed patients is 52 years, similar to the findings in this study.

Accounting for about 1.33% of rectal tumors, rectal carcinoids can give rise to non-specific symptoms such as general discomfort, constipation, blood in stool, abdominal pain, weight loss, and change in bowel habits[3]. Based on their origins, carcinoids can be divided into foregut, midgut and hindgut tumors. Rectal carcinoids, originating in the hindgut, generally secrete no or few active substances, such as 5-hydroxytryptamine (5-HT), resulting in normal 5-HT and 24 h 5-hydroxyindoleacetic acid levels and a very low risk of carcinoid syndromes in patients[4].

Colonoscopy is an important tool in the detection of rectal carcinoids. Typical manifestations include single or multiple yellow or gray submucosal nodular masses covered with normal mucosa. Atypical carcinoids are often associated with irregular, hard ulcers. Endoscopic ultrasonography has become a necessary part of the diagnosis of rectal carcinoids and helps to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis. Carcinoid lesions mostly originate in the submucosa or muscularis mucosa, and have a hypoechoic appearance. In addition, pathological examination is an important means of diagnosing rectal carcinoids. Typical histology includes solid nesting, nodular, insular, trabecular, palisading and rosette-like patterns, with small oval or polygonal, nested or glandular-like carcinoid cells, a regular and small nucleus, and little or no mitotic figures under the microscope. Chromogranin A, neuron-specific

enolase and synaptophysin are the most sensitive and specific markers of neuroendocrine cells[5].

Studies have shown that lymph node metastasis and liver metastasis of a given rectal carcinoid are significantly correlated with the size of the tumor. The incidence rates of lymph node metastasis are 3%, 7% and 64% for rectal carcinoids < 1 cm, 1-2 cm and > 2 cm in diameter, respectively. Therefore, it is generally believed that metastasis is rare in rectal carcinoids less than 1 cm, but more often seen in those > 2 cm[6]. Carcinoid metastasis occurs by direct invasion, including invasion of the surrounding tissue by serosal penetration, while lymph node metastasis or hematogenous metastasis can also be observed.

It is generally accepted that lymphatic invasion, muscularis propria invasion and lymph node metastasis are unlikely to occur in rectal carcinoid tumors < 1 cm in diameter and such small, well differentiated tumors can be removed endoscopically or by local excision. Endoscopic ultrasound must be employed to determine the exact size and depth of invasion of the tumor before surgery. The absence of preoperative staging will result in a suspicious or even positive pathological resection margin in 31.8%-83% of patients[7,8]. In contrast, appropriate endoscopic ultrasound staging before endoscopic submucosal dissection (ESD) reduces the postoperative positive margin rate to between 4.8% and 17%[9]. Therefore, this technique should be routinely performed before local excision of rectal carcinoids. Traditionally, most rectal carcinoid tumors of 1 cm or smaller are treated with EMR.

With the development of endoscopic techniques, many clinicians have been using ESD for the local resection of these tumors. Compared with traditional EMR, *en bloc* removal can be achieved using ESD regardless of tumor size, but it is also associated with a higher risk of complications (such as intraoperative or postoperative bleeding and perforation) and longer duration of operation. Yamaguchi *et al*[10] reported complete removal in 90% of their 20 patients with rectal carcinoids of < 1 cm using ESD, with an average operation time of 45 min, and one case of perforation. As reported by Lee *et al*[11], complete removal was achieved in 89.3% and 100% of patients undergoing EMR and ESD, without a significant difference in the size of tumors, respectively. EMR was successfully completed in all 21 patients with a rectal carcinoid of less than 1 cm in diameter without bleeding, perforation or other complications. The mean operation time was 15 min. The operation duration in the ESD group was significantly longer compared with the EMR group. It can be seen that, for lesions less than 1 cm in diameter, there is no considerable difference in the rate of complete removal between the EMR and ESD techniques, although a higher risk of complications and longer operation are associated with the latter technique.

For regional lymph node-negative patients with rectal carcinoids < 1 cm without muscular and vascular invasion, the 5-year survival rate can be 98.9% to 100% after treatment. Those who have positive lymph nodes but no distant metastases have a 5-year survival rate of 54% to 73%, and those with metastases have a 5-year survival rate of approximately 15%-30%[12]. Wang *et al*[13] found that the presence or absence of muscularis propria infiltration is the only determining factor in the 5-year survival rate, which is closely related to tumor size. Yoon *et al*[14] reported that, the possibility of distant metastasis from rectal carcinoids increases with tumor volume and T staging, as well as the presence of lymphatic, vascular or neural invasion. EMR was performed for the 21 patients with a rectal carcinoid of less than 1 cm in diameter. Pre-operative micro-probe ultrasound showed homogeneous hypoechoic masses derived from rectal mucosa and/or muscularis mucosa, but without infiltration in the muscularis propria or blood vessels. The tumor was completely removed. The resected specimens were immunohistochemically diagnosed as carcinoids. No relapse was noted during the follow-up of 1-2 years. The resection rate was high in our cohort and no relapse was noted, which may be explained by the small sample size and short follow-up. Therefore, studies with larger sample size and longer follow-up are warranted.

Although rectal carcinoid tumors are potentially malignant, they often grow slowly and present with a fairly long disease course, with favorable outcomes. To sum up, since lymphatic invasion, muscularis propria invasion and lymph node metastasis are unlikely to occur in rectal carcinoid tumors < 1 cm in diameter, these small, well differentiated tumors can be treated by endoscopic resection. A growing number of rectal carcinoids can be detected early with endoscopy, and endoscopic ultrasonography is a necessary part of the diagnosis of rectal carcinoids and can help to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis and choice of treatment. EMR and ESD are the primary endoscopic treatments for these conditions, and EMR is a safer option with fewer complications, shorter operating time, and comparable rate of complete removal to ESD. We believe that EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

COMMENTS

Background

Rectal carcinoids are rare neuroendocrine tumors that are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage. Rare as they are, carcinoids can occur in multiple body systems, with the gastrointestinal tract being the most commonly involved system. Rectal carcinoids are the third leading gastrointestinal carcinoids, accounting for about 14% of all conditions of this type, with an increasing incidence in recent years potentially attributable to extensive endoscopic application and increased awareness of carcinoid tumors.

Research frontiers

With the development of endoscopic techniques, many clinicians have been using endoscopic submucosal dissection (ESD) for the local resection of these tumors. Compared with traditional endoscopic mucosal resection (EMR), en bloc removal can be achieved using ESD regardless of tumor size, but it is also associated with a higher risk of complications (such as intraoperative or postoperative bleeding and perforation) and longer duration of operation.

Innovations and breakthroughs

A growing number of rectal carcinoids can be detected early with endoscopy,

and endoscopic ultrasonography is a necessary part of the diagnosis of rectal carcinoids and can help to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis and choice of treatment. EMR and ESD are the primary endoscopic treatments for these conditions, and EMR is a safer option with fewer complications, shorter operation time, and comparable rate of complete removal to ESD. The authors believed that EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

Applications

The authors evaluated the role of EMR under micro-probe ultrasound guidance in treatment of rectal carcinoids, and concluded that EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter.

Terminology

ESD is an advanced technique of therapeutic endoscopy for superficial gastrointestinal neoplasms. EMR represents a major advance in minimally invasive surgery in the gastrointestinal tract. EMR is based on the concept that endoscopy provides visualization and access to the mucosa, the innermost lining of the gastrointestinal tract.

Peer review

This is a retrospective case series of endoscopic removal of rectal carcinoid smaller than 1 cm. The authors evaluated the role of EMR under micro-probe ultrasound guidance in treatment of rectal carcinoids, and concluded that EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter. The paper is well organized and correctly reported. Some issues are needed to be concerned.

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META-ANALYSIS

Acid suppressive drugs and gastric cancer: A meta-analysis of observational studies

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Abstract

AIM: To evaluate the association between acid suppressive drug use and the development of gastric cancer.

METHODS: A systematic search of relevant studies that were published through June 2012 was conducted using the MEDLINE (PubMed), EMBASE, and Cochrane Library databases. The search included observational studies on the use of histamine 2-receptor antagonists (H_2RAs) or proton pump inhibitors and the associated risk of gastric cancer, which was measured using the adjusted odds ratio (OR) or the relative risk and 95%CI. An independent extraction was performed by two of the authors, and a consensus was reached.

RESULTS: Of 4595 screened articles, 11 observational studies (n = 94558) with 5980 gastric cancer patients were included in the final analyses. When all the studies were pooled, acid suppressive drug use was associated with an increased risk of gastric cancer risk (adjusted OR = 1.42; 95%CI: 1.29-1.56, I^2 = 48.9%, P = 0.034). The overall risk of gastric cancer increased among H_2RA users (adjusted OR = 1.40; 95%CI: 1.24-1.59, I^2 = 59.5%, P = 0.008) and PPI users (adjusted OR = 1.39; 95%CI: 1.19-1.64, I^2 = 0.0%, P = 0.377).

CONCLUSION: Acid suppressive drugs are associated with an increased risk of gastric cancer. Further studies are needed to test the effect of acid suppressive drugs on gastric cancer.

Key words: H2-receptor antagonists; Proton pump inhibitors; Gastric cancer; Meta-analysis

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INTRODUCTION

Acid suppressive drugs were the second most prescribed medication worldwide in 2005. Histamine 2-receptor antagonists (H_2RAs) and proton pump inhibitors (PPIs) were widely used for the treatment of peptic ulcers, gastroesophageal reflux disease (GERD), and other benign conditions of the stomach, esophagus, and duodenum[1-4]. PPIs were one of the most commonly prescribed medications by primary physicians and are frequently used

over the long term.

The safety of these drugs and their potential adverse effects is of great importance to public health. Several case reports suggested that acid suppressive drugs may increase the occurrence of gastric polyps or cancer[5-13], and several epidemiological studies have evaluated the association between long-term gastric acid suppression and the risk of gastrointestinal neoplasms. Several researchers have proposed that acid suppressive drugs may suppress gastric acid secretion and interfere with bacterial growth and nitrosamine formation[14-16]. In addition, the reduction of gastric acid secretion with acid suppressive drugs can lead to hypergastrinemia[17,18], which has been identified as a possible risk factor for gastric polyps and gastric and colonic carcinomas[19-22]. However, those findings are contradictory: several studies have found an increased risk of gastric cancer among acid suppressive drug users[23-25], whereas other studies found no evidence of an increased risk[26-28]. To date, no systematic meta-analysis has been published on this topic.

Therefore, in this study, we sought to investigate the association between the use of acid suppressive drugs and the risk of gastric cancer *via* a meta-analysis of cohort studies and case-control studies.

MATERIALS AND METHODS

Data sources and searches

Our review followed the Meta-analysis of Observational Studies in Epidemiology guidelines[29]. We performed our search in MEDLINE (PubMed) (inception to June 2012), EMBASE (inception to June 2012), and the Cochrane Library (inception to June 2012) using common key words regarding acid suppression and gastric cancer in case-control studies, cohort studies, and randomized controlled trials (RCTs). However, there were no RCTs among the search results that satisfied our inclusion criteria.

In addition, we searched the bibliographies of relevant articles to identify additional studies of interest. For the studies that did not directly report the association between the use of acid suppressive drugs and gastric cancer incidence, we contacted the authors in the field for any unpublished data. However, the authors did not have any available data to use in our meta-analysis. We used the following keywords in the literature search: "histamine receptor antagonist", "H_2 receptor antagonist", "cimetidine", "ranitidine", "famotidine", "nizatidine", "proton pump inhibitor", "proton pumps", "omeprazole", "nexium", "lansoprazole", "rabeprazole", "pantoprazole", or "esomeprazole" for the exposure factors and "stomach cancer", "stomach neoplasia", "gastric cancer", "gastric neoplasia", "stomach neoplasm" or "gastric neoplasm" for the outcome factors.

Study selection and data extraction

We included case-control studies and cohort studies that investigated the association between acid suppressive drug use and gastric cancer risk, which reported an adjusted odds ratio (OR) or relative risk (RR) and the corresponding 95%CI. We only selected articles that were written in English and excluded studies with no available data for outcome measures.

All the studies that were retrieved from the databases and bibliographies were independently evaluated by two authors of this paper (Ahn JS and Eom CS). Of the articles that were found in the three databases, duplicate articles and articles that did not meet the selection criteria were excluded. We extracted the following data from the remaining studies: the study names (first author), the year of publication, the country of publication, the study design, the study period, the population characteristics, the type of drugs, the adjusted OR or RR with a 95%CI: the study quality, and the adjustment. The data abstraction and the study selection were performed in duplicate.

Quality assessment

We assessed the methodological quality of the included studies using the Newcastle-Ottawa Scale (NOS) for the case-control and cohort studies in the meta-analysis[30]. The NOS is comprehensive and has been partially validated for assessing the quality of non-randomized studies in meta-analyses. The NOS is based on the following three broad subscales: the selection of the study groups (4 items), the comparability of the groups (1 item), and the ascertainment of the exposure and the outcome of interest for case-control studies and cohort studies, respectively (3 items). A "star system" (a score range from 0-9) has been developed for quality assessment. Each study can be awarded a maximum of one star for each numbered item within the selection and exposure categories, whereas a maximum of two stars can be assigned for comparability. In this study, we considered a study that was awarded 7 or more stars as a high-quality study because standard criteria have not been established.

Statistical analysis

The outcome of the meta-analysis was the risk of gastric cancer. We used the adjusted data (adjusted OR or RR with a 95%CI) for the meta-analysis. In addition, we conducted subgroup analyses by type of study design (case-control studies *vs* cohort studies), type of acid suppressive drugs (H_2RAs *vs* PPIs), duration of acid suppressive drug use (within 5 years *vs* more than 5 years), location of gastric cancer (gastroesophageal junction, cardia, or non-cardia), and study quality (high-quality *vs* low-quality).

We pooled the adjusted ORs, RRs and 95%CIs based on both fixed-effects and random-effects models. Because the incidence of gastric cancer was low (< 5%), as evidenced in the cohort studies, we concluded that the outcome was sufficiently rare to assume that the OR could be used to approximate the RR. Heterogeneity was assessed using the Higgins I^2 value, which measures the percentage of total variance across studies, which is attributable to heterogeneity rather than chance. Negative

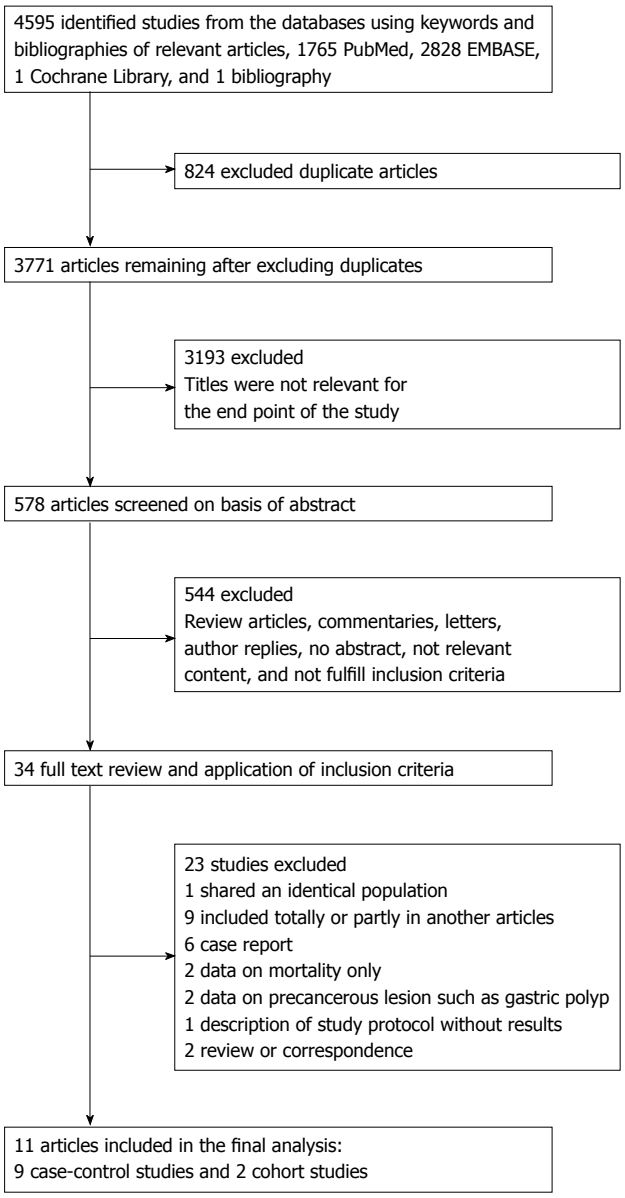

Figure 1 Flow diagram for identification of relevant studies.

I^2 values were set at zero to ensure that I^2 fell between 0% (no observed heterogeneity) and 100% (maximal heterogeneity)[31,32]. We considered an I^2 value greater than 50% to represent substantial heterogeneity. When the heterogeneity was substantial, we conducted sensitivity analyses: the changes in the I^2 values were examined by removing each study from the analysis to determine which studies contributed most significantly to the heterogeneity. We assessed between-group heterogeneity using analysis of variance. When heterogeneity was found, we performed a meta-regression using the subgroup categories.

We used the Woolf method (inverse variance method) for a fixed-effects analysis[33,34], and we used the DerSimonian and Laird method for a random-effects analysis[35]. Begg's funnel plot and Egger's test were used to identify publication bias. Publication bias was detected in studies when the funnel plot was asymmetrical or the *P*-value was less than 0.05 using Egger's test. We used the Stata/SE version 10.1 software program (StataCorp, College Station, TX, United States) for the statistical analysis.

RESULTS

Identification of relevant studies

Figure 1 shows a flow diagram of the study selection. A total of 4595 articles were identified by searching the three databases and relevant bibliographies. We excluded 824 duplicate articles and 3737 articles that did not satisfy the selection criteria. After the full texts of the remaining 34 articles were reviewed, the following 23 articles were excluded: one article from a study that shared an identical population[36]; 6 articles that were case reports[5-10]; 2 articles that included data on precancerous lesions, such as gastric polyps[37,38]; one article that described a study protocol without results[39]; 2 articles that only included mortality data[40,41]; 2 articles that were reviews or correspondence[25,42]; and 9 articles that were included totally or partially in another article[43-51]. As a result, we included 11 observational studies (9 case-control studies and 2 cohort studies), which ultimately met our inclusion criteria.

Characteristics of the studies included in the final analysis

Table 1 shows the main characteristics of the 11 reviewed studies. Five studies were published in the 1990s[23-27], and 6 studies were published in the 2000s[52-57]. The countries where the studies had been conducted were as follows: the United States (n = 5), the United Kingdom (n = 2), Denmark (n = 2), Canada (n = 1), and Italy (n = 1). We identified a total of 94558 participants, which included 5980 cases and 88578 controls from the following articles: 1 article on a medical record-based case-control study, 3 articles on nested case-control studies, 2 articles on population-based case-control studies, and 2 articles on prospective cohort studies. Seven studies evaluated the association between H_2RA use and the risk of gastric cancer, and 4 studies assessed the association between the use of H_2RAs or PPIs and the risk of gastric cancer. The mean value for the methodological quality of the 11 studies was 6.64 stars according to the NOS (Table 1).

Overall use of acid suppressive drugs and the risk of gastric cancer

Figure 2 shows the association between the use of acid suppressive drugs and gastric cancer risk. The overall use of H_2RAs and PPIs was associated with an increased risk of gastric cancer in 9 case-control studies [adjusted OR = 1.36; 95%CI: 1.23-1.50, n = 9, I^2 = 24.5% (range, 0.0%-64.0%)] and in 2 cohort studies [adjusted RR, 2.01; 95%CI: 1.51-2.68, n = 2, I^2 = 63.0% (not available)] using a fixed-effects model, and this association was observed in a combined study [adjusted OR and RR, 1.42; 95%CI: 1.29-1.56, n = 11, I^2 = 48.9% (range, 0.0%-74.0%)]. As shown in Table 2, an increased risk of gastric cancer was found with H_2RA use [adjusted OR = 1.40; [95%CI: 1.24-1.59, n = 10, I^2 = 59.5% (range, 19.0%-80.0%)] and with PPI use [adjusted OR = 1.39; 95%CI: 1.19-1.64,

Table 1 Characteristics of observational studies on acid suppressive drugs and gastric cancer

Ref.	Country	Design	Study period	Range of age (yr)	Population	Drugs	Adjusted OR or RR (95%CI)	Study quality[1]	Adjustment for covariates
La Vecchia *et al*[23]	Italy	CC	1985-1989	27-74	563 cases and 1501 controls	Cimetidine, Ranitidine	1.80 (1.20 -2.70)	5	Age, sex, education, smoking, alcohol, coffee
Johnson *et al*[24]	United States	CC	1988-1992	NA	113 cases and 452 matched controls	Cimetidine, Ranitidine	2.00 (1.00 -3.90)	5	Age, sex, first pharmacy use
Møller *et al*[25]	Denmark	PCC	1977-1990	NA	134 cases among 16739	Cimetidine	2.30 (1.60-3.10)	6	Age, sex, diagnosis, method of diagnosis
Schumacher *et al*[26]	United States	CC	1981-1987	17-94	99 cases and 365 controls	Cimetidine	2.30 (0.80 -6.90)	6	Age, sex, date of first pharmacy use
Chow *et al*[27]	United States	MCC	1986-1992	NA	196 cases and 200 controls	Cimetidine, Ranitidine, Famotidine, Nizatidine	0.60 (0.30-1.30)	6	Race, smoking, BMI, number of composite conditions[2]
Farrow *et al*[52]	United States	PBCC	1993-1995	30-79	293 esophageal adenoca, 221 esophageal SCC, 261 gastric cardia adenoca, 368 non-cardia gastric adenoca and 695 controls	Cimetidine, Ranitidine, Famotidine, Nizatidine	1.00 (0.60-1.90)	7	Age, sex, study center, smoking, BMI, history of gastric or duodenal ulcer, GERD symptom frequency, alcohol
Suleiman *et al*[53]	United Kingdom	NCC	1990-1992	NA	231 cases among 9876 controls	Cimetidine, Ranitidine	2.56 (0.17-38.09)	7	Age, sex, MI, antacid, steroid, smoking, alcohol, social class, height, weight
García Rodríguez *et al*[54]	United Kingdom	NCC	1994-2001	40-84	287 esophageal adenoca, 195 gastric cardiac adenoca, 327 gastric non-cardia adenoca and 10000 controls	H_2RAs, PPIs	1.24 (0.88-1.75)	8	Age, sex, calendar year, smoking, alcohol, BMI, UGI disorder, hiatal hernia, GU, DU, dyspepsia
Tamim *et al*[55]	Canada	NCC	1995-2003	NA	1589 cases and 12991 controls	H_2RAs, PPIs	1.37 (1.22-1.53)	8	Age, sex number of prescriptions to any drug, total length of hospitalizations and number of visit to GPs, specialist, emergency rooms
Duan *et al*[56]	United States	PBCC	1992-1997	NA	220 esophageal adenoca, 277 gastric cardiac adenoca, 441 distal gastric adenoca and 1356 controls	H_2RAs, PPIs	1.15 (0.58 -2.29)	7	Age, sex, race, birthplace, education, smoking, BMI, history of UGI symptom
Poulsen *et al*[57]	Denmark	PCC	1990-2003	NA	161 cases among users of 18790 PPIs or 17478 H_2RAs	Omeprazole, Lanzoprazole, Esomeprazole, Pantoprazole, Rabeprazole, Cimetidine, Ranitidine, Nizatidine, Famotidine	1.30 (0.70-2.30)	8	Age, sex, calendar period, history of *H.pylori*, gastroscopy year, COPD, alcohol, NSAID

[1]Study quality was judged based on the Newcastle-Ottawa Scale (range, 1-9 stars). The mean value for the methodological quality of 11 studies was 6.64 stars; [2]Number of composite conditions were created to include gastroesophageal reflux, hiatal hernia, esophagitis/esophageal ulcer, or difficulty swallowing. OR: Odds ratio; RR: Relative risk; CC: Case-control; MCC: Medical record based case-control; PBCC: Population-based case-control study; NCC: Nested case-control study; PCC: Prospective cohort study; NA: Not available; Adenoca: Adenocarcinoma; SCC: Squamus cell carcinoma; H_2RAs: Histamine 2-receptor antagonists; PPIs: Proton pump inhibitors; BMI: Body mass index; GERD: Gastroesophageal reflux disease; MI: Myocardial infarction; UGI: Upper gastrointestinal; GU: Gastric ulcer; DU: Duodenal ulcer; GPs: General practitioners; COPD: Chronic obstructive pulmonary disease; NSAID: Nonsteroidal anti-inflammatory drug.

n = 3, I^2 = 0.0% (range, 0.0%-90.0%)]. In a sensitivity analysis, when the study by Møller *et al*[25] was removed, the I^2 values of the H_2RA studies decreased from 59.5% to 34.3%, but the effect remained significant. In addition, when stratified by the study design, H_2RA use was positively associated with gastric cancer in both case-control [adjusted OR = 1.30; 95%CI: 1.13-1.49, n = 8, I^2 = 42.0% (range, 0.0%-74.0%)] and cohort [adjusted RR, 1.84; 95%CI: 1.41-2.41, n = 2, I^2 = 80.4% (not available)] studies. The use of PPIs was associated with an increased risk of gastric cancer in case-control studies [adjusted OR = 1.44; 95%CI: 1.21-1.71, n = 2, I^2 = 23.5% (not available)], whereas only one cohort study was conducted to evaluate the use of PPIs (adjusted RR, 1.20; 95%CI: 0.80-1.80) (data were not shown).

Subgroup meta-analysis

In a subgroup meta-analysis, acid suppressive drugs were associated with an increased gastric cancer risk within 5 years of use [adjusted OR = 1.58; 95%CI: 1.35-1.81,

Table 2 Association between acid suppressive drugs use and gastric cancer risk in subgroup meta-analysis

Category	No. of studies	Adjusted OR/RR (95%CI)	Heterogeneity, I^2 % (95%CI)	Model used	Heterogeneity between groups
Type of drugs					P = 0.01
H_2RAs	10[23-27,52-55,57]	1.40 (1.24-1.59)	59.5 (19.0-80.0)[1]	Fixed-effects	
PPIs	3[54,55,57]	1.39 (1.19-1.64)	0.0 (0.0-90.0)	Fixed-effects	
Location of gastric cancer					P = 0.03
GE junction	2[24,26]	2.28 (0.97-5.35)	0.0 (NA)	Fixed-effects	
Gastric cardia	4[52,54-56]	0.88 (0.63-1.24)	9.2 (0.0-86.0)	Fixed-effects	
Non-cardia	6[24,26,52,54-56]	1.42 (1.12-1.79)	0.0 (0.0-75.0)	Fixed-effects	
Duration of drugs use					P = 0.27
Within 5 yr	7[23,24,26,27,52,55,57]	1.58 (1.35-1.81)	60.2 (9.0-83.0)[1]	Fixed-effects	
Over 5 yr	6[23,24,26,27,52,57]	1.24 (0.84-1.84)	25.4 (0.0-69.0)	Fixed-effects	
Study quality					P = 0.01
High-quality[2]	6[52,53,54,55-57]	1.34 (1.21-1.48)	0.0 (0.0-75.0)	Fixed-effects	
Low-quality	5[23-27]	1.86 (1.49-2.32)	63.5 (4.0-86.0)[1]	Fixed-effects	

[1]I^2 value greater than 50% represents substantial heterogeneity. We conducted sensitivity analyses by removing each study to determine the studies that contributed most to the heterogeneity; [2]High quality study was considered a study awarded 7 or more stars, as standard criteria have not been established. OR: Odds ratio; RR: Relative risk; H_2RAs: Histamine 2-receptor antagonists; PPIs: Proton pump inhibitors; GE junction: Gastroesophageal junction; NA: Not available.

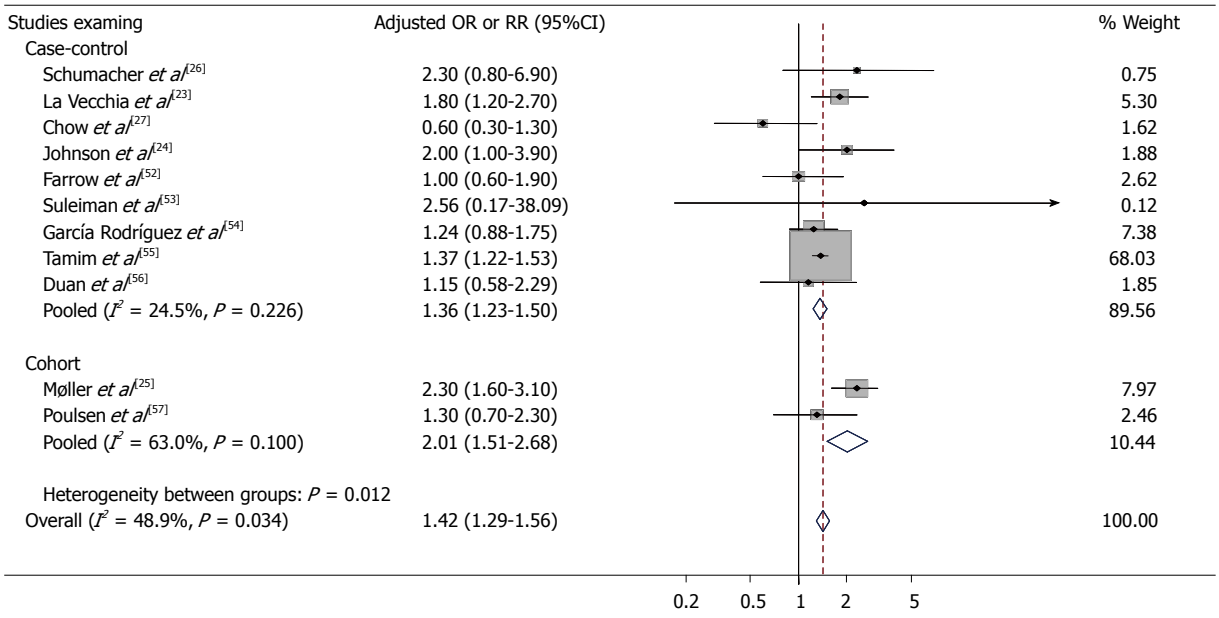

Figure 2 Meta-analysis results of individual and pooled adjusted odds ratio or relative risk of gastric cancer. The size of each square is proportional to the study's weight. Diamonds are the summary estimate from the pooled studies with 95%CI. OR: Odds ratio; RR: Relative risk.

n = 7, I^2 = 60.2% (not available)]. In a sensitivity analysis, when the study by La Vecchia *et al*[23] was removed, the I^2 values decreased from 60.2% to 41.4%; however, the summary estimate indicated an elevated risk of gastric cancer. Additionally, a positive association was observed in patients with more than 5 years of acid suppressive drug use. However, there was no statistical significance [adjusted OR = 1.24; 95%CI: 0.84-1.84, n = 6, I^2 = 25.4% (not available)] (Table 2). The between-group differences in the effect estimates for within 5 years of exposure *vs* more than 5 years of exposure were not significant (P = 0.27).

Regarding the location of gastric cancer, a significant positive association was observed between the use of acid suppressive drugs and non-cardia cancer risk [adjusted OR = 1.42; 95%CI: 1.12-1.79, n = 6, I^2 = 0.0% (not available)], whereas a marginal significance was observed in gastroesophageal junction cancer and the use of acid suppressive drugs [adjusted OR = 2.28; 95%CI: 0.97-5.35, n = 2, I^2 = 0.0% (not available)]. However, there was no significant association between the use of acid suppressive drugs and gastric cardia cancer [adjusted OR = 0.88; 95%CI: 0.63-1.24, n = 4, I^2 = 9.2% (0.0%-86.0%)]. The between-group differences in the effect estimates for non-cardia cancer *vs* gastroesophageal junction cancer *vs* gastric cardia cancer were significant (P = 0.03). The meta-regression according to the site of cancer indicated that the effect estimates for non-cardia cancers were significantly higher than those for cardia cancers (P = 0.02), and the effect estimates for gastroesophageal junction

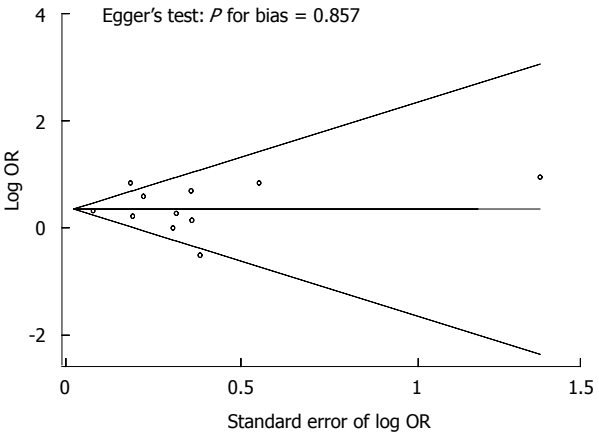

Figure 3 Begg's funnel plots and Egger's test for identifying publication bias (*P* = 0.857) in a meta-analysis of observational studies (*n* = 11). OR: Odds ratio.

cancers were significantly different from those of cardia cancers ($P = 0.04$).

There was an increased risk of gastric cancer that was associated with the use of acid suppressive drugs in both high-quality [adjusted OR = 1.34; 95%CI: 1.21-1.48, $n = 6$, $I^2 = 0.0\%$ (range, 0.0%-75.0%)] and low-quality [adjusted OR = 1.86; 95%CI: 1.49-2.32, $n = 5$, $I^2 = 63.5\%$ (range, 4.0%-86.0%)] studies. No publication bias was observed in the selected studies (Figure 3, Begg's funnel plot was symmetrical; Egger's test, P for bias = 0.857).

DISCUSSION

In this meta-analysis of 11 observational studies, we found that both H_2-receptor antagonist use and proton pump inhibitor use were associated with an increased risk of gastric cancer. In site-specific analyses, an increased risk of non-cardia gastric cancer was observed in patients who used acid suppressive drugs, whereas acid suppressive drug use was not associated with the risk of gastric cardia cancer.

Our meta-analysis has several strengths. This systemic review was the most comprehensive meta-analysis to date of observational studies that addressed the association between the use of acid suppressive drugs and the risk of gastric cancer, which included a large number of studies and participants. We performed a detailed analysis by stratifying the type of drugs (H_2RAs or PPIs), the location of gastric cancer (gastroesophageal junction, cardia, or non-cardia), the duration of acid suppressive drug use, and the study quality.

Previous studies suggested that long-term H_2RA use may increase gastric cancer[11-13], and long-term PPI treatment induced gastric fundic polyps, which led to the development of precancerous lesions[5-8]. There is biological evidence of the effect of acid suppressive drug use on the risk of gastric cancer. First, PPIs and H_2RAs can reduce gastric acidity by modulating H(+)-K(+)-ATPase or competitive inhibitors of histamine binding sites in gastric parietal cells. Decreased gastric acidity, whether caused by gastric atrophy or acid suppressive drug-induced hypochlorhydria, may result in increased bacterial colonization and a greater number of bacteria that can produce nitrosamines[16,58], which are compounds that are associated with an increased risk of gastric adenocarcinoma[59]. Second, the reduction of gastric acid secretion by acid-suppressive drugs switches on the positive feedback of a gastric acid-producing cascade, which leads to hypergastrinemia[19]. This condition is a possible cause of carcinoids, gastric polyps, and gastric and colonic carcinomas because elevated serum gastrin could have a trophic effect on neoplastic growth in the gastrointestinal tract[60]. The use of long-term PPIs can cause hyperplasia in enterochromaffin-like cells and increase the incidence of atrophic gastritis and gastric polyps, which are a precursor to gastric cancer[37,38,43-46].

We found that acid suppressive drugs increased the risk of non-cardia gastric cancer, whereas acid suppressive drug use was not associated with a risk of gastric cardia cancer. One possible mechanism may be associated with the augmented effects of acid suppressive drugs and other risk factors of non-cardia gastric cancer, such as gastric atrophy and *Helicobacter pylori* (*H. pylori*) infection[61,62]. Several authors suggested that omeprazole treatment was associated with an elevated incidence of gastric corpus mucosal atrophy[63], and long-term PPI treatment in *H. pylori* infected patients could accelerate the development of corpus atrophic gastritis[64,65]. In addition, recent studies suggested that a history of *H. pylori* eradication prior to long-term PPI therapy could prevent the development of atrophic gastritis[57,66,67]. Considering this knowledge, further studies are needed to clarify the association between acid suppressive drug use and the risk of non-cardia gastric cancer, especially for patients with gastric atrophy and *H. pylori* infection.

We found that within 5 years of use, acid suppressive drugs increased the risk of gastric cancer, whereas there was a non-significant increased risk of gastric cancer among users of acid suppressive drugs for more than 5 years. Our meta-analysis was underpowered to detect statistically significant differences between the studies according to the duration of exposure; however, the qualitative differences were noteworthy. Early gastric symptoms of stomach cancer are typically similar to those of benign conditions, such as peptic ulcers, GERD, or functional gastrointestinal disease[23], which could lead to the use of acid suppressive drugs. Therefore, we could not exclude the possibility of misspecification and the protopathic bias of gastric cancer, especially among 5-year acid suppressive drug users. We found that two of the earliest studies (Møller *et al*[25] and La Vecchia *et al*[23]) contributed most significantly to the heterogeneity in the studies of H_2RAs and in the studies that examined exposure within 5 years of the incidence of cancer. These studies may have been influenced by misspecification because endoscopic tools that would have detected early stage cancer were not readily available when the studies were conducted. There could be bias due to existing gastric conditions; however, we recommend that future

studies carefully consider the appropriate control of previous gastric conditions.

Our meta-analysis has several limitations. First, most of the studies in our meta-analysis were observational studies. Observational studies, even when well-controlled, are susceptible to various biases, which may reduce the quality of the analysis. Second, most of the studies in our meta-analysis came from Western countries. The occurrence of gastric cancer is rapidly increasing in the United States and in Western Europe[68]; however, this disease is a more significant public health problem in Asia. Third, we did not have access to individual data on dose-response relationships that may have affected gastric acid production. Finally, we could not evaluate the effect of underlying gastric conditions, *e.g.*, *H. pylori* infection, because these data were not presented in each study.

Our meta-analysis demonstrated that the use of acid suppressive drugs was associated with an increased risk of gastric cancer. Our findings should be confirmed by more prospective cohort studies that are designed with larger sample sizes and longer follow-up durations to test the effect of acid suppressive drugs on the risk of gastric cancer. These studies should focus on previous underlying gastric conditions for which acid suppressive drugs are prescribed and the dose response of acid suppressive drugs use.

COMMENTS

Background

The widespread use of acid suppressive drugs has led to concern about the development of adverse effects owing to prolonged gastric acid suppression, particularly the development of gastric polyps or gastric neoplasms. Authors performed a meta-analysis of cohort studies and case-control studies to determine whether the use of acid suppressive drugs, such as histamine 2-receptor antagonists (H_2RAs) and proton pump inhibitors (PPIs), can increase the risk for gastric cancer.

Research frontiers

Several case reports suggested that antacids may increase the risk of gastric polyps or cancer. Several epidemiological studies have evaluated the association between long-term gastric acid suppression and the risk of gastrointestinal neoplasms. However, epidemiological studies have reported inconsistent findings regarding the association between the use of acid suppressive drugs and gastric cancer risk. To date, no systematic meta-analysis has been published on the use of acid suppressive drugs and the risk of gastric cancer.

Innovations and breakthroughs

In this meta-analysis of case-control and cohort studies, the authors found that both H_2RA and PPI use were associated with an increased risk of gastric cancer. In site-specific analyses, an increased risk of non-cardia gastric cancer was observed in patients who used acid suppressive drugs, whereas acid suppressive drug use was not associated with a risk of gastric cardia cancer.

Applications

The use of acid suppressive drugs is associated with an increased risk of gastric cancer. However, authors could not exclude the possibility of misspecification and the protopathic bias of gastric cancer. The early gastric symptoms of stomach cancer are typically similar to those of benign conditions, such as peptic ulcers, gastroesophageal reflux disease, or functional gastrointestinal disease, which could lead to the use of acid suppressive drugs. There could be bias due to existing gastric conditions; therefore, they recommend that future studies carefully consider the appropriate control of previous gastric conditions.

Terminology

Acid suppressive drugs can reduce gastric acidity by modulating H(+)-K(+)-ATPase or competitive inhibitors of histamine binding sites in gastric parietal cells, which leads to hypergastrinemia. This condition is a possible cause of carcinoids, gastric polyps, and gastric and colonic carcinomas because elevated serum gastrin could have a trophic effect on neoplastic growth in the gastrointestinal tract.

Peer review

This study may lead conclusion that acid suppressive drugs such as H_2R blocker or PPI may act as a stimulator for gastric cancer. It is difficult to obtain a substantial conclusion from 11 studies adopted by this paper because all 11 papers were observational study and no information on *Helicobacter pylori* infection. This paper is good for publication.

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CASE REPORT

Portal vein stenosis after pancreatectomy following neoadjuvant chemoradiation therapy for pancreatic cancer

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Abstract

Extrahepatic portal vein (PV) stenosis has various causes, such as tumor encasement, pancreatitis and as a post-surgical complication. With regard to post-pancreaticoduodenectomy, intraoperative radiation therapy with/without PV resection is reported to be associated with PV stenosis. However, there has been no report of PV stenosis after pancreatectomy following neoadjuvant chemoradiation therapy (NACRT). Here we report the cases of three patients with PV stenosis after pancreatectomy and PV resection following gemcitabine-based NACRT for pancreatic cancer and their successful treatment with stent placement. We have performed NACRT in 18 patients with borderline resectable pancreatic cancer since 2005. Of the 15 patients who completed NACRT, nine had undergone pancreatectomy. Combined portal resection was performed in eight of the nine patients. We report here three patients with PV stenosis, and thus the ratio of post-operative PV stenosis in patients with PV resection following NACRT is 37.5% in this series. We encountered no case of PV stenosis among 22 patients operated with PV resection for pancreatobiliary cancer without NACRT during the same period. A relationship between PV stenosis and NACRT is suspected, but further investigation is required to determine whether NACRT has relevance to PV stenosis.

Key words: Pancreatic cancer; Portal vein stenosis; Neoadjuvant chemoradiation therapy; Pancreatectomy; Expandable metallic stent

Core tip: Intraoperative radiation therapy for pancreatic cancer with/without portal vein (PV) resection is reported to be associated with PV stenosis. However, there has been no report of PV stenosis after pancreatectomy following neoadjuvant chemoradiation therapy (NACRT). Here we report the cases of three patients with PV stenosis after pancreatectomy and PV resection following gemcitabine-based NACRT for pancreatic cancer and their successful treatment with stent placement. We have performed pancreatectomy with PV resection following NACRT in 8 patients with borderline resectable pancreatic cancer since 2005. The ratio of post-operative PV stenosis is 37.5% in this series.

Tsuruga Y, Kamachi H, Wakayama K, Kakisaka T, Yokoo H, Kamiyama T, Taketomi A. Portal vein stenosis after pancreatectomy following neoadjuvant chemoradiation therapy for pancreatic cancer. *World J Gastroenterol* 2013; 19(16): 2569-2573 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i16/2569.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i16.2569

INTRODUCTION

Extrahepatic portal vein (PV) stenosis can occur due to

tumor encasement[1], pancreatitis[2] and as a post-surgical complication-especially post-liver transplantation[3]. With regard to post-pancreaticoduodenectomy (PD), intraoperative radiation therapy (IORT) with/without PV resection is reported to be associated with PV stenosis[4-6]. The incidence rate for PV stenosis is reported to be 11% to 23%[5,6].

Portal hypertension secondary to PV stenosis causes gastrointestinal bleeding from gastroesophageal or jejunal varices, and refractory ascites[1]. Gastrointestinal bleeding is the most serious life-threatening complication. Refractory ascites is not fatal but affects the patient's quality of life.

Here we provide the case reports of three patients with PV stenosis after pancreatectomy and PV resection following neoadjuvant chemoradiation therapy (NACRT) for pancreatic cancer, and we discuss the relationship between PV stenosis and NACRT and the indications for stent placement. To the best of our knowledge, PV stenosis after pancreatectomy following NACRT has not been described in the literature.

CASE REPORT

Case 1

A 54-year-old man underwent PD and PV resection for pancreatic cancer following NACRT. The protocol consisted of external-beam radiotherapy to the pancreatic bed and regional lymphatics for a total dose of 50.4 Gy in 28 fractions. Concomitant chemotherapy consisted of gemcitabine at a dose of 150 mg/m^2 once weekly. The reconstruction between the PV and the superior mesenteric vein (SMV) was end-to-end anastomosis using a continuous running suture of 6-0 prolene. The splenic vein was not reconstructed.

Refractory ascites and malnutrition were recognized at 9 postoperative months (POMs). Computed tomography (CT) showed short segmental stenosis of the PV in the region of the anastomosis, severe ascites, and liver atrophy (Figure 1). Intraoperative portography through the catheter via the ileocolic vein and balloon dilation were performed but were not sufficiently effective because the region had elastic stenosis. Therefore, an expandable metallic stent (EMS; 1 cm diameter, 3 cm length) was inserted into the stenotic region. The portal venous pressure decreased from 14.5 to 8.6 cm H_2O, and the pressure gradient of 6.5 cm H_2O across the PV stenosis disappeared. Anticoagulant therapy was initiated immediately after stent placement. Heparin was administered at a dose of 10000 IU/d by intravenous infusion for 3 d initially, and then oral warfarin was administered. The warfarin was switched to aspirin 3 mo later. After the stent placement, a follow-up CT showed that the patient's ascites decreased and his liver atrophy improved (Figure 2). Stent patency is maintained at present, 5 years after the placement.

Case 2

A 44-year-old man underwent distal pancreatectomy and

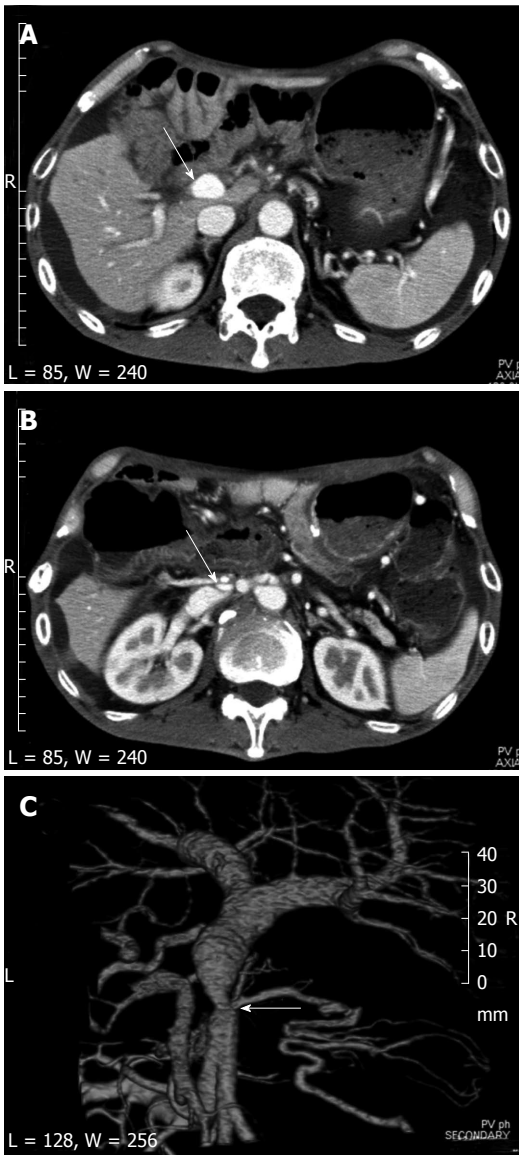

Figure 1 Computed tomography showed short segmental stenosis of the portal vein in the region of the anastomosis, severe ascites, and liver atrophy. A, B: Computed tomography (CT) scan shows severe ascites and liver atrophy (arrow) (A), and stenosis of the portal vein (arrow) behind the superior mesenteric artery (B); C: The image of the 3D reconstruction of the portal vein shows short segmental stenosis in the region of the anastomosis (arrow).

PV resection simultaneously with splenectomy and total gastrectomy for pancreatic cancer following NACRT using the same protocol as that described for Case 1. The PV was preoperatively occluded by tumor thrombus, and a cavernous transformation was identified. The reconstruction between the PV and the SMV was end-to-end anastomosis using the same procedure as that described for Case 1. Refractory diarrhea, ascites and malnutrition were recognized at 5 POMs. Initially, malabsorption was suspected, and thus total parenteral nutrition support was initiated. There was no improvement in symptoms after the improvement in nutrition status. CT showed short segmental stenosis of the PV in the region of the anastomosis, collateral circulation through the cavernous transformation of the pancreatic head, severe ascites, and thickness of the intestinal wall (Figure 3).

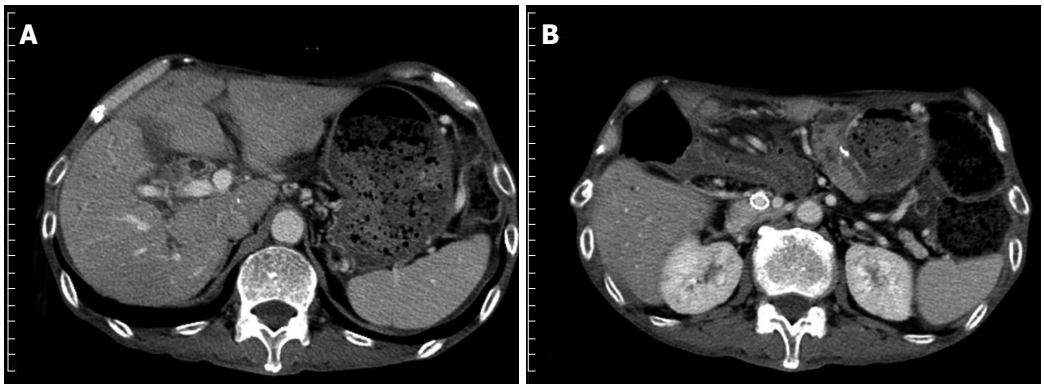

Figure 2 Computed tomography scan 3 mo after the expandable metallic stent placement. A: Shows that the ascites decreased and the liver atrophy improved; B: The stent placed in the portal vein remained patent.

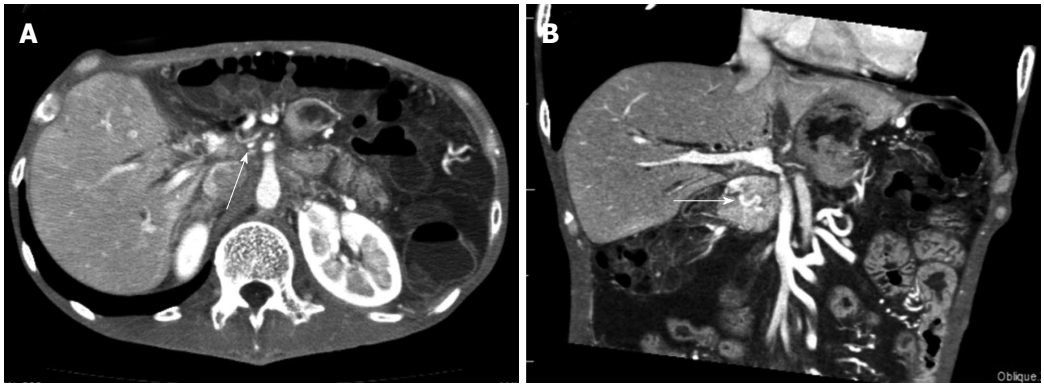

Figure 3 Computed tomography showed short segmental stenosis of the portal vein in the region of the anastomosis, collateral circulation through the cavernous transformation of the pancreatic head, severe ascites, and thickness of the intestinal wall. A: Computed tomography scan showing severe portal vein stenosis (arrow) in the region of the anastomosis; B: Multiplanar reconstruction revealed the collateral circulation through the cavernous transformation of the pancreatic head (arrow), severe ascites and thickness of the intestinal wall.

We suspected portal hypertension secondary to PV stenosis, even though the portal venous flow seemed to be sustained by the collateral circulation. Percutaneous transhepatic direct portography was performed (Figure 4A). The portal venous pressure was 31.0 cm H_2O, and the pressure gradient across the PV stenosis was 21.0 cm H_2O. An EMS (1 cm diameter, 4 cm length) was inserted into the stenotic region (Figure 4B). The portal venous pressure decreased to 17.0 cm H_2O, and the pressure gradient decreased to 2.0 cm H_2O. Anticoagulant therapy was performed as that described for Case 1 After a stent placement, the patient's diarrhea and ascites improved. Stent patency is maintained at present, 1.5 years after the placement.

Case 3

A 68-year-old woman underwent PD and PV resection for pancreatic cancer following NACRT using the same protocol as that described for Case 1. The reconstruction between the PV and the SMV was end-to-end anastomosis using the same procedure as that described for Case 1. Malnutrition and ascites were recognized at 5 POMs. Total parenteral nutrition support and repeated drainage of ascites were performed, but there was no improvement of the ascites. Cytology of the ascites showed no evidence of malignancy. CT showed short segmental stenosis of the PV in the region of the anastomosis. Percutaneous transhepatic direct portography was performed (Figure 4C). The portal venous pressure was 24.5 cm H_2O, and the pressure gradient across the PV stenosis was 12.0 cm H_2O. An EMS (1 cm diameter, 3 cm length) was inserted into the stenotic region (Figure 4D). The portal venous pressure decreased to 11.5 cm H_2O, and the pressure gradient disappeared. Anticoagulant therapy was performed as that described for Case 1. After the stent placement, the patient's ascites improved. Stent patency is maintained at present, 4 mo after the placement.

DISCUSSION

Despite considerable research, the prognosis for pancreatic cancer remains poor. For all stages combined, the 1- and 5-year relative survival rates are 25% and 6%, respectively[7]. Complete surgical resection is the only therapy to afford a chance of cure[8], but patients with borderline resectable pancreatic cancer are at high risk of having positive surgical margins due to vascular involvement[9]. NACRT for borderline resectable pancreatic cancer is expected to increase the margin-negative resection rate and improve survival[10].

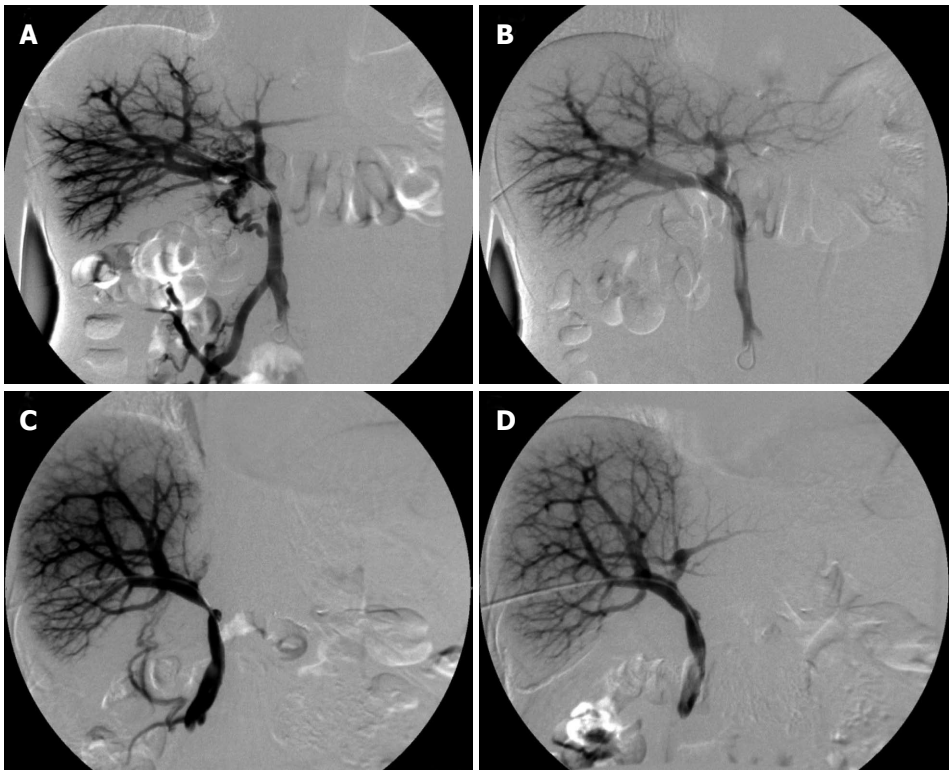

Figure 4 Percutaneous transhepatic direct portography showing short segmental stenosis of the portal vein in the region of the anastomosis. A: Collateral circulation of the pancreatic head; B: After the expandable metallic stent (EMS) placement, the stenosis was improved, and the collateral circulation disappeared; C: The blood flow of the umbilical portion of the left portal vein was unclear; D: The stenosis was improved, and the blood flow of the umbilical portion became clear after the EMS placement.

Table 1 The clinical characteristics of three patients

Pt. No.	Age (yr)	Sex	Operative procedure	Symptoms	Months before onset	Procedure of stent placement	Pressure gradient (cmH_2O)		Improvement in symptoms
							Before	After	
1	54	M	PD	Ascites, malnutrition	9	Intraoperative, via ileocolic vein	6.5	0	Yes
2	44	M	DP	Ascites, malnutrition, diarrhea	5	Percutaneous transhepatic	21.0	2.0	Yes
3	68	F	PD	Ascites, malnutrition	5	Percutaneous transhepatic	12.0	0	Yes

PD: Pancreaticoduodenectomy; DP: Distal pancreatectom; M: Male; F: Female.

We have performed gemcitabine-based NACRT in 18 patients with borderline resectable pancreatic cancer since 2005. Of 15 patients who completed the NACRT, nine had pancreatectomy. Combined portal resection was performed in 8 of the 9 patients. We report here the cases of three patients with PV stenosis (Table 1), and thus the ratio of post-operative PV stenosis in patients with PV resection following NACRT increases to 37.5% in our patient series.

There was no case of PV stenosis among 22 patients who underwent PV resection for pancreatobiliary cancer without NACRT in the same period in our department. The incidence of PV obstruction after PD with PV resection has been reported as 1.5%[11] and 25%[12]. The incidence of PV stenosis after PD with PV resection is rarely reported. Leach *et al*[12] reported the incidence 18% (5 of 29 patients), but the 18% includes 14 patients who received IORT. Mitsunaga *et al*[5] suggested that the mechanism of the development of extrahepatic PV occlusion after IORT is associated with the periportal changes induced by IORT and periportal fibrosis. We did not find any reports of PV stenosis after pancreatectomy following NACRT for pancreatic cancer because most papers about NACRT for pancreatic cancer report only perioperative complications[13,14]. However, it seems possible that the periportal changes are induced by neoadjuvant radiation, similar to those induced by IORT, and they increase the risk of the development of PV stenosis.

The first choice of treatment for PV stenosis after liver transplantation is balloon dilation[15]. The indication of stent placement is limited to elastic stenosis and recurrent stenosis. Case 1 had an elastic stenosis, and thus the EMS placement was done after the venoplasty. However, we placed the stents in Cases 2 and 3 before the

venoplasty. Placing a stent for benign stenosis before a venoplasty is controversial. Takaki *et al*[16] speculated that stent placement is essential for the treatment of early anastomotic stenosis because such stenosis is caused by reactive edema or technical problems, and balloon angioplasty fails to dilate the vessel due to recoil. The onset of the stenosis in the three cases presented here was also early (5-9 POMs). Considering the poor prognosis of pancreatic cancer, early improvement of a patient's symptoms and quality of life is important.

In Case 1, we placed the stent *via* ileocolic vein because massive ascites interfered with transhepatic puncture. However, in Cases 2 and 3, we could safely performed percutaneous transhepatic stent placement after draining the ascites.

In conclusion, we have reported the cases of three patients with PV stenosis after pancreatectomy and PV resection following NACRT for pancreatic cancer. A relationship between PV stenosis and NACRT is suspected, but further investigation is required to determine whether NACRT has relevance to PV stenosis.

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CASE REPORT

Henoch-Schonlein purpura with intestinal perforation and cerebral hemorrhage: A case report

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Abstract

Henoch-Schonlein purpura (HSP) with intestinal perforation and cerebral hemorrhage is a very rare clinical condition. There has been no report of HSP complicated with both intestinal perforation and cerebral hemorrhage until October 2012. Here we describe a case of HSP with intestinal perforation and cerebral hemorrhage in a 5-year-old girl. Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm. Partial resection of the small intestine was performed, and pathological analysis suggested chronic suppurative inflammation in all layers of the ileal wall and mesentery. Seventeen days after surgery, cerebral hemorrhage developed and the patient died.

Key words: Henoch-Schonlein purpura; Anaphylactoid purpura; Small intestine; Cerebral hemorrhage; Child

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INTRODUCTION

Henoch-Schonlein purpura (HSP), also known as anaphylactoid purpura, is the most common form of systemic vasculitis in children[1]. It occurs most commonly in children between 3 and 10 years old[2] and affects small vessels. Typical symptoms of HSP include palpable purpura, arthralgia, stomach ache and hematochezia. Pathologically, HSP is characterized by the development of leukocytoclastic vasculitis in small vessels with deposition of immune complexes mainly consisting of immunoglobulin A. Although the etiology is still unclear, HSP is known to develop after upper respiratory infection or drug allergy[3] and is therefore considered an autoimmune disease. It is reported that the incidence of HSP with intestinal perforation is 0.38%, mainly affecting the ileum and occasionally the jejunum, where intestinal perforation appears about 10 d after the onset of initial symptoms[4]. The incidence of HSP with central nervous system symptoms is between 0.9% and 6.9%, and HSP with intracranial hemorrhage has an even lower incidence[5]. The most common sign of HSP with intracranial hemorrhage is seizure, followed by headache, changes in mental status, and focal neurologic deficits[6]. To date, there have been only 10 reported cases of HSP with cerebral hemorrhage. Herein we report a case of HSP with intestinal perforation and cerebral hemorrhage.

CASE REPORT

A 5-year-old Chinese girl was admitted to our intensive care unit with complaints of abdominal pain for 22 d, rash for 21 d, and hematuria for 2 wk. She was previously healthy and had a history of allergy to aminopyrine.

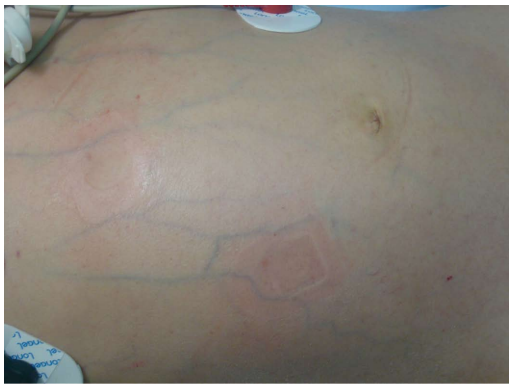

Figure 1 Abdominal bulge was found on examination at admission.

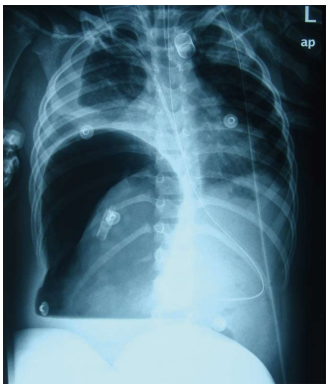

Figure 2 Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm.

Upon physical examination, her general condition was poor. Her blood pressure was 112/69 mmHg, heart rate 153 beats/min, body temperature 37.2 ℃, and arterial oxygen saturation 87%. She was unconscious and presented with difficulty breathing, cyanotic lips, rales, abdominal bulge (Figure 1), marked abdominal tenderness, rebound tenderness and muscle tension, and marked edema of the bilateral lower extremities up to the level of her knees. She had purpuric papules, 1-2 cm in diameter, over her ankles, buttocks and lower legs, as well as raised flesh-colored papules over her upper medial legs, but her upper trunk and perineum were not involved. Neurologic exam was within normal limits. Laboratory data on admission were as follows: white blood cell count 45900/μL with an increase in the percentage of neutrophils (93.5%); hemoglobin 69 g/L; hematocrit 20.9%; platelet count 40000/L; prothrombin time 10.6 [international normalized ratio (INR) 0.99]; serum total protein 33 g/L; albumin 12 g/L; cholinesterase 223 U/L; alkaline phosphatase 128 U/L; urea 15.3 mmol/L; serum creatinine 84.0 μmol/L; urine protein 2+; and urine occult blood 5+. Cerebral and chest computed tomography (CT) demonstrated slight widening of cerebral sulci and cisterns, inflammation of the lungs, and bilateral pleural effusions. Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm (Figure 2). Abdominal ultrasonography showed remarkable intestinal expansion in the lower abdomen and increased bilateral renal artery resistance index. To improve the patient's condition, symptomatic treatments were given, including immediate intubation, mechanical ventilation, improvement of circulation, correction of disturbances of the internal environment, and application of broad-spectrum antibiotics. On January 13, the girl underwent emergency laparotomy. Intraoperative findings included necrosis of the intestine (10-30 cm long), with multiple perforations visible from the terminal ileum to the ileocecal valve, severe intestinal adhesion, and the presence of a large number of pus mosses on the surface of the liver, intestine and peritoneum. Surgical treatment strategies were enterectomy, enterostomy and peritoneal lavage. Postoperative pathological analysis suggested chronic suppurative inflammation in all layers of the ileal wall and mesentery (Figure 3). Thus, the patient was diagnosed with Henoch-Schönlein purpura complicated by intestinal necrosis and perforation, nephritis, respiratory failure, and severe sepsis.

Postoperatively, comprehensive treatments were given, including broad-spectrum antibiotics, gamma-globulin, hormones, nutritional support, and mechanical ventilation. Following these treatments, the girl's condition showed significant improvement: her inspired oxygen concentration was 40% and arterial oxygen saturation was 97%; the tension of the abdominal wall was reduced; and the surgical incision healed well. Apart from blood pressure (maintained at 130/90 mmHg) that was not controlled effectively by antihypertensive therapy, all other vital signs were satisfactory. Laboratory data after surgery were as follows: white blood cell count 24700/μL with an increased percentage of neutrophils (93.3%); hemoglobin 69 g/L; hematocrit 20.9%; platelet count 157000/L; prothrombin time 12 (INR 1.29); serum total protein 38 g/L; albumin 19 g/L; urea 13.2 mmol/L; serum creatinine 100 μmol/L; urine protein 2+; and urine occult blood 3+.

On January 28, the patient became unconscious suddenly and developed ptosis and mydriasis. Cerebral CT showed subarachnoid hemorrhage, right thalamic and intraventricular hemorrhage, and multiple ischemic changes in the left cerebellar hemisphere and bilateral cerebral hemispheres (Figure 4). Despite active rescue, the girl died on January 28.

DISCUSSION

HSP is a systemic vasculitis which affects small vessels, with typical symptoms including palpable purpura, arthralgia, stomach ache and bloody stools. Pathologically, HSP is characterized by the development of leukocytoclastic vasculitis in small vessels with deposition of immune complexes consisting mainly of immunoglobulin A[3]. Although all HSP patients invariably develop cutaneous purpura, other symptoms, such as arthritis, abdominal pain, and renal involvement, do not occur in all patients, which make the diagnosis of HSP more challenging[7]. In a few cases, HSP can affect other systems or organs and

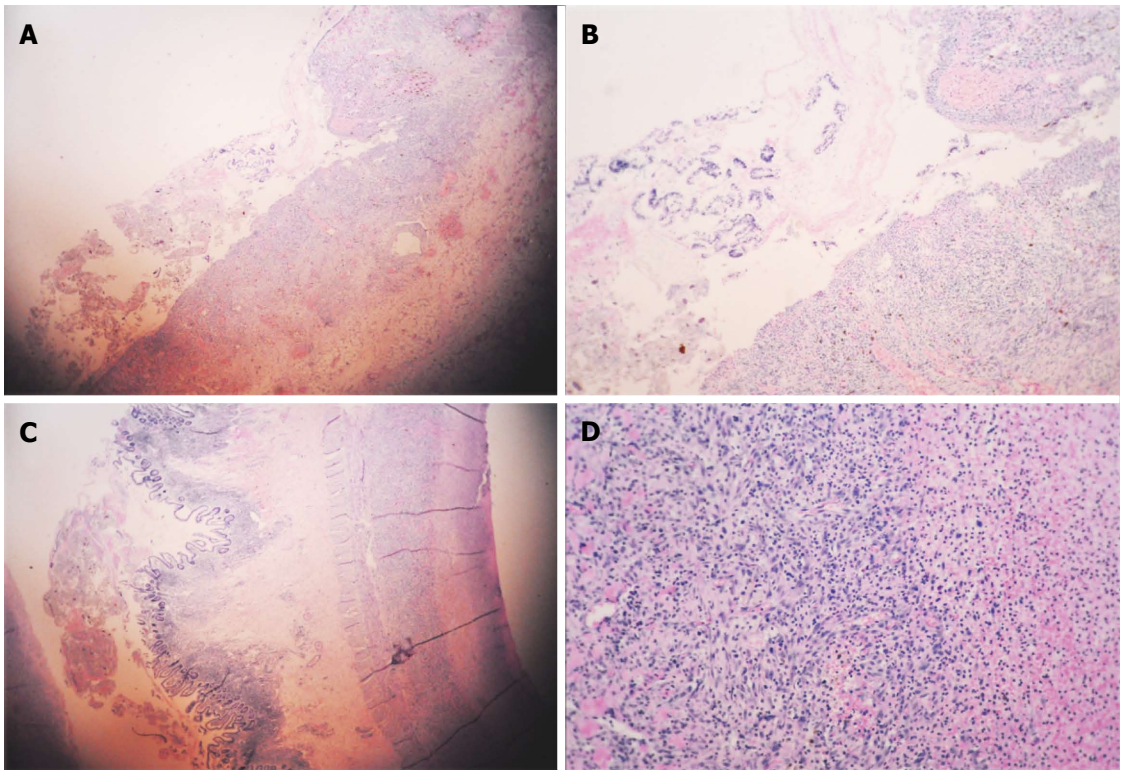

Figure 3 Histopathological analyses of the patient's ileal biopsy specimens. A: Hematoxylin and eosin-stained sections of the biopsy specimens revealed mucosal discontinuity (magnification × 10); B: Multiple site bleeding (magnification × 20); C: Inflammatory cell infiltration in all layers of the ileal wall (magnification × 20); D: Infiltration of a large number of neutrophils and cell deformation in the area of edema (magnification × 40).

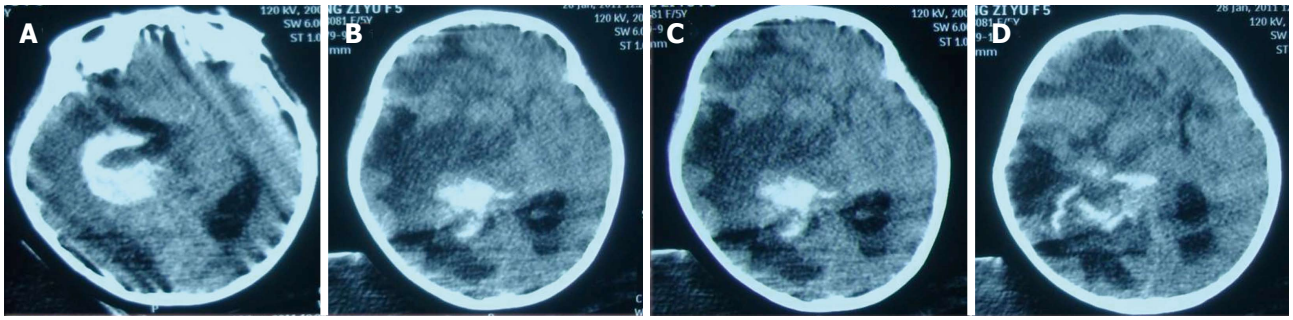

Figure 4 Cerebral computed tomography demonstrated subarachnoid hemorrhage, right thalamic and intraventricular hemorrhage, and multiple ischemic changes in the left cerebellar hemisphere and bilateral cerebral hemispheres. A: Right thalamic hemorrhage ruptured into the ventricle; B-D: Thalamic hemorrhage complicated with subarachnoid hemorrhage, cerebral hemispheres multiple focal ischemia.

results in the occurrence of transient paralysis, convulsions, myocarditis, hypertension, and other symptoms[4]. Although palpable purpura is often considered the hallmark of HSP, skin findings were not described as the presenting feature in nearly 25% of patients[8].

Gastrointestinal manifestations are present in 45% to 75% of patients with HSP, with 14% to 36% of patients developing these symptoms prior to the appearance of purpura[9], as occurred in our case. After being treated with corticosteroids, gastrointestinal lesions in HSP are usually reversible and can heal, although a few patients (2%-6%) develop conditions requiring surgery[10]. It is reported that approximately 52% of HSP patients have varying degrees of gastrointestinal bleeding, mostly self-limiting; however, cases with serious intestinal bleeding (0%-8.2%) call for intervention, and misdiagnosis can be life-threatening[11]. Patients most commonly develop abdominal pain without complications. The most commonly reported complication is intussusception[12,13], while bowel ischemia and infarction, perforation, stricture, fistula, hemorrhage, pancreatitis, and appendicitis are infrequent. In our case, heavy gas in the right subphrenic space with an elevated diaphragm was detected radiographically, and surgical treatment strategies were enterectomy, enterostomy and peritoneal lavage. This observation may be of great clinical significance, as these gastrointestinal manifestations are usually serious and can result in invasive diagnostic techniques, including laparotomy. The incidence of HSP with central nervous system symptoms ranges from 0.9% to 6.9%, and HSP with intracranial hemor-

rhage has an even lower incidence[5]. The most common sign of HSP with intracranial hemorrhage is seizures, followed by headache, changes in mental status, and focal neurologic deficits. HSP as a form of necrotizing vasculitis results in a wide range of pathologic manifestations. According to the severity and site of the vascular lesions, diffuse encephalopathy, focal ischemia, or intracranial hemorrhages may develop[6,14]. With respect to the causes of cerebral hemorrhage in our case, the primary disease may play a major role because it can affect small vessels and lead to systemic vasculitis. Interestingly, we found the coexistence of cerebral ischemia and cerebral hemorrhage. It may be assumed that immunoglobulin A immune complex deposition initiates arteriolar inflammation in the cerebral vasculature as well as in the systemic vessels[12,14]. Cerebral hemorrhage was possibly secondary to resistant hypertension and cerebral vasculitis. Both purpura nephritis and increased bilateral renal artery resistance index could lead to resistant hypertension. In addition, cerebral hemorrhage might be associated with cerebral vascular malformation[5]; however, this possibility could not be ruled out in our case because of postoperative mechanical ventilation.

In conclusion, we herein describe a case of intestinal perforation and cerebral hemorrhage secondary to HSP. Treatment of the primary disease is very important in preventing the occurrence of serious complications, such as intestinal perforation and cerebral hemorrhage. HSP patients should be closely monitored for early serious complications such as intestinal perforation and given timely surgery. Surgery is the most effective treatment. Despite its rarity, HSP should be considered when gastrointestinal perforation is observed. In addition, blood pressure should be controlled effectively in HSP patients to prevent the occurrence of serious neurological complications such as cerebral hemorrhage.

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CASE REPORT

Surgical treatment of a patient with peliosis hepatis: A case report

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Abstract

This report describes a case of a space-occupying lesion in the right liver in a 38-year-old man who was found to have peliosis hepatis. Clinical data of this patient were presented, including medical history, laboratory test and imaging results, and postoperative pathological findings (hematoxylin and eosin staining). Review of his medical history showed that the patient had been bitten by a dog three years earlier. B-mode ultrasonography revealed an uneven echo mass in the right hemiliver, and magnetic resonance imaging scans also showed a mass in the anterior segment of the right liver. Upon surgical removal, the mass was found to be 4.0 cm × 3.8 cm × 3.8 cm in size and located in segment Ⅵ. The mass had a dark and soft appearance, with an irregular edge on intraoperative ultrasonography. Postoperative pathological findings revealed many small capsules filled with blood cells. The patient was diagnosed with peliosis hepatis based on his medical history of having been bitten by a dog, presence of mild anemia, and lack of characteristic symptoms, including fever of unknown origin, abdominal pain, and hepatosplenomegaly, combined with intraoperative and postoperative pathologic findings. The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

Key words: Peliosis hepatis; Surgical treatment; Ultrasonography; Space-occupying lesion

Core tip: This report describes a case of a space-occupying lesion in the right liver in a 38-year-old man who was diagnosed with peliosis hepatis based on the medical history of having been bitten by a dog, presence of mild anemia, and lack of characteristic symptoms, including fever of unknown origin, abdominal pain, and hepatosplenomegaly, combined with intraoperative and postoperative pathologic findings. The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

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INTRODUCTION

Peliosis hepatis (PH) is a rare clinical disease, appearing mostly as diffuse hepatic lesions. PH is considered a benign vasogenic lesion and is characterized by the presence of cystic blood-filled cavities distributed randomly throughout the liver parenchyma. This disease is more

common in adults than in children or adolescents. We herein report a case of PH in a 38-year-old man.

CASE REPORT

A 38-year-old man presented with a space-occupying lesion in his right liver. The patient had been bitten by a dog three years earlier, but his health was generally good. The patient did not have hepatosplenomegaly or symptoms of tuberculosis intoxication, nor did he have a history of blood transfusions or long-term use of glucocorticoids or anabolic hormones. Physical examination revealed no abnormalities. Blood tests showed that his hemoglobin level was 90 g/L, whereas his blood biochemistry, liver and kidney function tests, and alpha fetal protein, cancer embryo antigen, carbohydrate antigen 19-9 concentrations were all within normal ranges. He was positive for hepatitis B surface antibody (323.21 IU/L) and core antibody (4.25 S/C.0), but negative for other hepatitis B antigens and antibodies and negative for human immunodeficiency virus. Hepatobiliary magnetic resonance imaging (MRI) revealed a 3.8 cm × 3.1 cm mass with irregular boundaries in the anterior segment of his right liver.

Enhanced scanning showed inhomogeneous enhancement, peaking at the arterial phase (Figure 1). Color ultrasonography showed that his liver had a normal appearance, but there was a 3.9 cm × 3.9 cm mass with inhomogeneous echo in his right hemiliver with unclear boundaries and weak blood flow signal within the mass (Figure 2). He underwent surgery, which revealed the mass located in segment Ⅵ. Intraoperative ultrasonography showed that the mass was 4.0 cm × 3.8 cm × 3.8 cm in size, and had a dark and soft appearance, with irregular edges (Figure 3). No abnormality was observed in any other abdominal organs. Rapid pathological examination on frozen sections suggested the possibility of a hepatic hemangioma, although pathological assessment confirmed PH (Figure 4). The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

DISCUSSION

At gross inspection, the peliotic lesions in a PH liver look like "Swiss cheese slices". Microscopically, there are two types of peliosis: "parenchymal peliosis" and "phlebectatic peliosis"[1], the main difference being whether the cavities are lined by endothelium or fibrotic tissue. The patient described here had parenchymal peliosis.

The pathogenesis of PH remains unknown, and pathogenic factors vary. Roughly they can be divided into three categories: drug-related, autoimmune and infectious. PH has been associated with the use of hormones and immunosuppressive medications, especially α-alkyl steroid hormones and thiopurine[2]. Autoimmune factors are those associated with secondary immunodeficiency caused by certain potential consumptive diseases, such as tuberculosis, hematological malignancies[3,4], acquired immunodeficiency syndrome[5], immune deficiency after transplantation[6], and hepatocellular carcinoma (HCC)[7]. Infectious factors include Bartonella infection, which leads to cat-scratch disease[8,9]. PH has also been observed in dogs infected with Bartonella[10]. Despite the absence of symptoms of cat-scratch disease, the patient's dog-bite experience suggests the possibility of Bartonella infection.

PH is usually caused by autoimmune disorders triggered by endogenous and exogenous risk factors. These autoimmune disorders can induce primary dysfunction in the endothelial cells of the liver sinus, as well as hepatocyte necrosis. This causes angiectasis and hyperemia, producing multiple blood-filled cystic spaces in the liver parenchyma.

Clinical development of PH is not apparent, and it is difficult to detect at its onset. The diagnosis of PH is based on pathological examination and liver imaging. Imaging can disclose either intrahepatic space-occupying or diffuse lesions. MRI scans of the patient reported here showed a mass in the anterior segment of the right liver, approximately 3.8 cm × 3.1 cm in size, with irregular boundaries, that showed a weak T1 and a strong T2 signal, resulting in inhomogeneous enhancement. In addition, the diffusion weighted imaging phase showed a strong signal with increasing B values. This result is consistent with those observed in other patients with PH[11].

PH shows a broad spectrum of appearances on radiography, primarily because the demonstrations of MRI scans largely depend on the blood supply to the lesions[12]. This lack of specificity can easily result in a misdiagnosis of other hypervascular tumors[13]. In addition, PH and HCC may occur together[7]. Therefore, only pathologic findings are considered the gold standard for the diagnosis of PH.

PH is a benign vasogenic lesion, which usually can be cured by removal of the mass or part of the liver. No patient has shown recurrence or metastasis. Its special pathological structure nonetheless makes patients prone to liver rupture and hemorrhage[14] or death due to hepatic failure. The incidence of PH is relatively low, but it has high risks. Thus, it should be aggressively treated once diagnosed, in order to prevent complications. Treatment should be tailored based on the location and extent of the lesion, the damage to liver function, and whether or not serious amalgamative complications can occur.

Because of its insidious onset and insufficient specific clinical manifestations, the diagnosis of PH is often delayed, making treatment more difficult. Most patients are not diagnosed until PH develops into diffuse lesions accompanied by severe liver failure, at which point liver transplantation is life-saving[15]. The treatments for diffuse lesions without severe damage to liver function typically include finding and removing the cause (*i.e.*, treating the primary disease, controlling infection, or stopping any associated drugs), assisted by medicines that protect liver function. Liver transplantation is indicated, how-

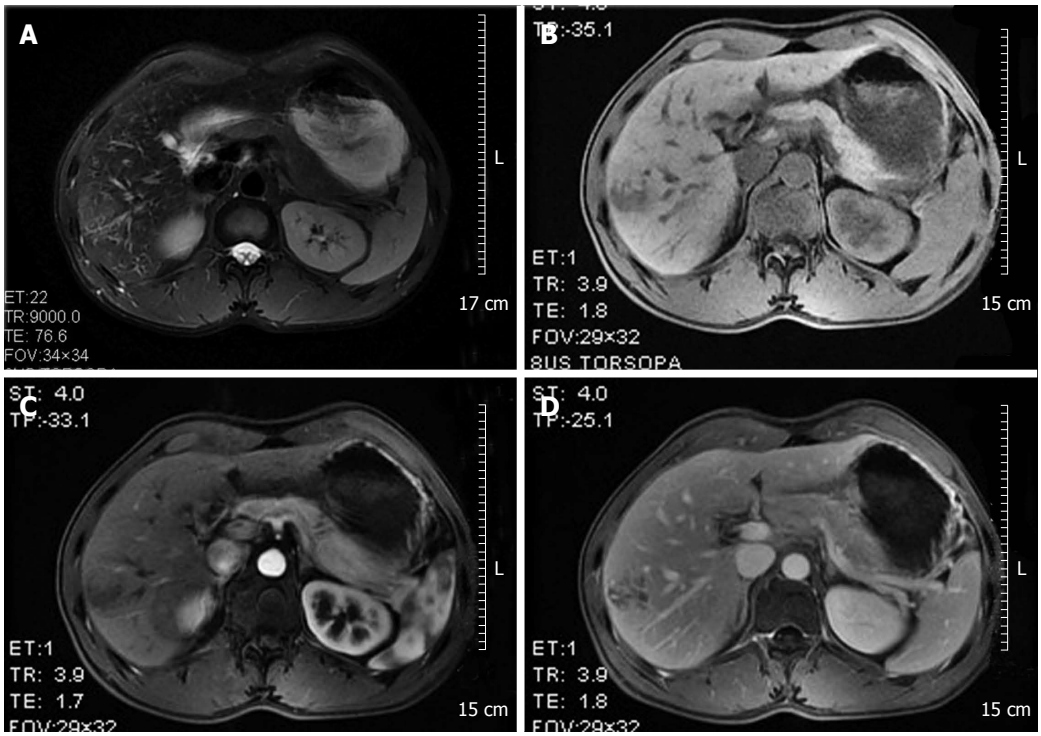

Figure 1 Magnetic resonance imaging scans. A: Hepatobiliary magnetic resonance imaging scans revealed a 3.8 cm × 3.1 cm mass in the anterior segment of the right liver with irregular boundaries, producing low-intensity T1 signals and high-intensity T2 signals; B-D: Gadolinium diethylenetriamine-pentaacid enhanced scanning showed inhomogeneous enhancement, peaking during the arterial phase and declining during the portal vein and parenchymal phases. The lumen and collateral vessels of the portal vein were well defined without filling defects.

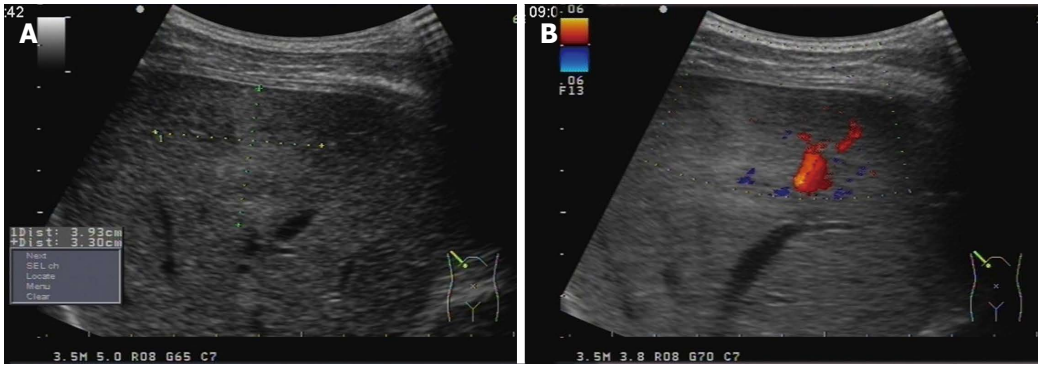

Figure 2 B-mode ultrasonography. A: B-mode ultrasonography showing a 3.9 cm × 3.3 cm mass with inhomogeneous echo in the right hemiliver; B: The boundary was unclear and some blood-flow signal was detected within the mass.

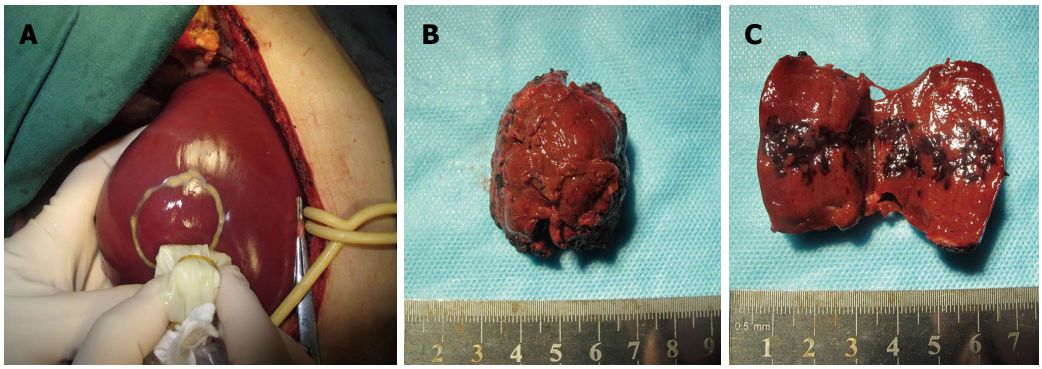

Figure 3 Gross appearance of the mass after surgical removal. A: There were no clear boundaries or lining membrane between the mass and the liver; B: The specimen had an irregular shape; C: Several blood-filled cystic spaces were observed after opening the mass.

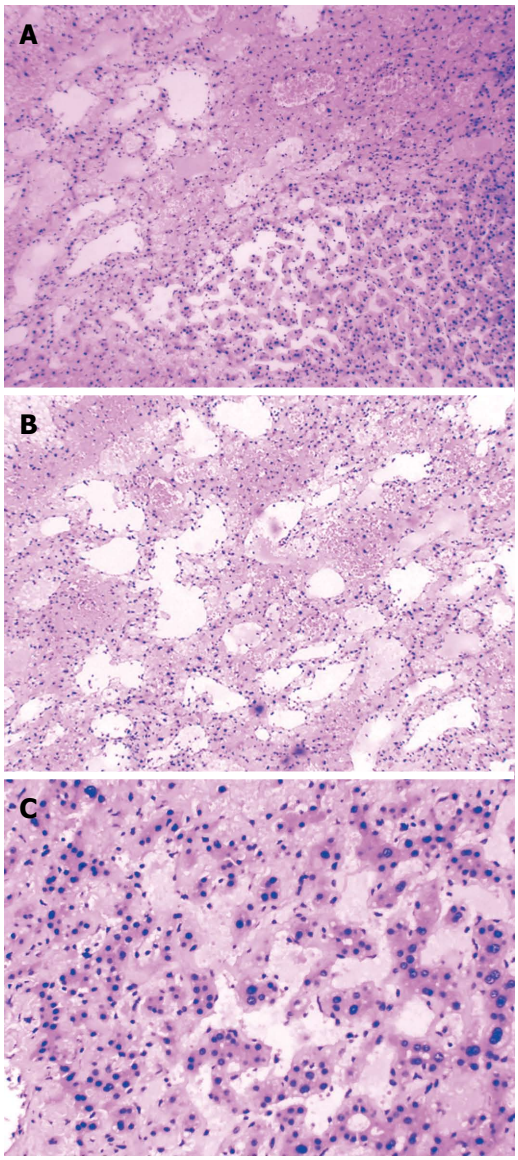

Figure 4 Pathologic examination, showing irregular and dilated lacunae, some of which were filled with blood and without endothelial linings. Hematoxylin and eosin staining, original magnification A: × 100; B: × 200; C: × 400.

ever, for patients with severely diffuse lesions accompanied by hepatic failure. If focal lesions are diagnosed by laparoscopy, biopsy or fine-needle aspiration and the cause has been clearly determined, the treatment usually consists of removing the cause and closely monitoring the patient. Patients with ruptured lesions and bleeding should be promptly treated by transcatheter superselective embolization or even surgical hemostasis (if necessary)[16,17]. If the cause is unknown or conservative treatment is ineffective, surgery should be performed. Although the lesion was focal in the patient described in this report, its space-occupying nature could not be clearly estimated. The patient had no liver function abnormality (Child-Pugh grade A) and no relevant basic disease or medical history. However, the patient had mild anemia that might be caused by hemorrhage of the lesion. Since surgical removal can effectively prevent complications such as abdominal bleeding, and can result in a clear diagnosis to guide further treatments, we decided to perform a precise hepatectomy. The long-term therapeutic effects remain to be determined.

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Role of molecular analysis in the adjuvant treatment of gastrointestinal stromal tumours: It is time to define it

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Abstract

Sendur *et al* pointed out the attention on the importance of mutational analysis for adjuvant treatment of gastrointestinal stromal tumor (GIST) in an article published in *World Journal of Gastroenterology*. In particular, they suggested that the optimal dose and duration of adjuvant therapy could be defined by the mutational status of the primary disease. This comment would underline the importance of centralised laboratories, given the increasingly important role of molecular analysis in the work-flow of all GIST, and the need of retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting, in order to define the role of mutational analysis in choosing the optimal dose and duration of adjuvant therapy.

Key words: Gastrointestinal stromal tumours; Platelet-derived growth factor receptor alpha; KIT; Wild-type; Molecular analysis; Imatinib; Adjuvant treatment

Core tip: Sendur *et al* pointed out the attention on the importance of mutational analysis for adjuvant treatment of gastrointestinal stromal tumor (GIST). In particular, they suggested that the optimal dose and duration of adjuvant therapy could be defined by the mutational status of the primary disease. This topic represents a big challenge in GIST's management by now, because even if the molecular analysis is strictly recommended in the work-flow of all GIST, its role in the adjuvant setting remains still unsettled due to the lack of prospective large clinical trials. In particular we pointed out the attention on the KIT/platelet-derived growth factor receptor alpha wild type GIST, that are extremely heterogeneous both in clinical and molecular background, making difficult their management also in the adjuvant setting. Our comment would underline the importance of centralised laboratories, given the increasingly important role of molecular analysis in the work-flow of all GIST, and the need of retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting, in order to define the role of mutational analysis in choosing the optimal dose and duration of adjuvant therapy.

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TO THE EDITOR

We read with great interest the article by Sendur *et al*[1] entitled "Is exon mutation analysis needed for adjuvant treatment of gastrointestinal stromal tumor?", that has been recently published in the January issue (2013) of *World Journal of Gastroenterology*. The authors pointed out the likely importance of mutational analysis for guiding

the clinicians in the treatment's choice of gastrointestinal stromal tumor (GIST) patients also in the adjuvant setting[1]. In particular they suggest that the optimal dose and duration of adjuvant treatment could be defined by the mutational status of the primary disease. Their account comes from the consolidated evidence that the mutation status is predictive of response to imatinib treatment in the advanced disease[2-5]. In fact it is well known that *KIT* exon 11 mutations are associated to the highest response rate to imatinib in comparison to patients exon 9 mutations[2,3]. On the contrary exon 18 *PDGRFA* D842V point mutation confers primary resistance to imatinib[4,5]. Furthermore it has been widely shown that the subgroup of patients with *KIT* exon 9 mutation treated with imatinib 800 mg had an higher progression-free survival in comparison with those treated with the standard dose of 400 mg[6]. Translating these evidences from the advanced disease to the limited disease the authors suggested that it may be reasonable to assume that patients with a substantial risk of GIST relapse harbouring *KIT* exon 9 mutation should be considered for higher dose of adjuvant imatinib.

Moreover, in the last years the potential role of mutational status as a prognostic factor has been progressively appeared[7]. In particular, it has been shown that deletions of *KIT* exon 11, especially those involving codon 557 and/or codon 558 are associated with a shorter progression-free and overall survival whereas most platelet-derived growth factor receptor alpha (*PDGFRA*)-mutant GISTs generally have a lower potential for malignancy[8-15].

By now even if the optimal duration of adjuvant imatinib therapy is still unclear, adjuvant imatinib for three years of duration as a standard of care in high-risk operable GISTs is recommended[16,17].

Whether the role of mutational status as a prognostic factor should be confirmed in large series, the authors suggest that the optimal duration of adjuvant treatment could be different in relation to the kind of mutation found and not only to the standard prognostic factors.

Based on the above considerations, the mutational analysis is strictly recommended in the work-flow of all cases of primary GIST, because it provides information about tumour sensitivity to imatinib and, although not yet included in any risk stratification system, it may provide prognostic information[17-19]. However, the decision-making on adjuvant therapy treatment based on mutational analysis alone is not still supported by consistent data, with the exception of *PDGFRA* D842V mutation[17].

In particular, the adjuvant therapy in the *KIT*/*PDGFRA* wild type (WT) GIST, which notably may be less sensitive to imatinib than most mutated GIST, remains controversial. In fact, in recent years is emerging more and more the idea that *KIT*/*PDGFRA* WT GIST should be considered as a heterogeneous group of disorders rather than a single molecular subtype of GIST, both in clinical behaviour and molecular background[20-27]. For example, it has been recently identified a subgroup of *KIT*/*PDGFRA* WT GIST, characterized by germline and somatic mutations in succinate dehydrogenase (*SDH*) subunits B, C and A and defined in different ways as *SDH*-decifient GIST, or type-2 or pediatric-type GIST[24-27]. These patients have in common several pathological and clinical features, such as the epitheliod pattern, the multifocal presentation, the female prevalence, the gastric primary tumor localization, and the indolent course of disease despite the presence of lymph nodes and liver metastases up-front and independently to standard prognostic parameters. Moreover it seems that they have also a questionable sensitivity to imatinib. Given their indolent behaviour when metastatic, *KIT*/*PDGFRA* WT GIST *SDH*-deficient may not benefit from adjuvant treatment irrespective to the standard risk stratification, whereas more aggressive *KIT*/*PDGFRA* WT GIST without *SDH*-impairment, may be probably considered as all mutated GIST.

Therefore also the effect of adjuvant imatinib on *KIT*/*PDGFRA* WT GIST may be variable and clinical decision-making should be individualised case by case taking into account various molecular data and shared with the patient[17].

In conclusion, given the increasingly important role of molecular analysis in the work-flow of all GIST, centralised laboratories should be widely warranted. Furthermore, the special attention pointed up by the authors on the "optimal dose" and the "duration" of adjuvant treatment defined by the mutational status of the primary disease should be used at first for the decision to suggest or not the imatinib treatment in this setting. Finally, since prospective clinical trials with large series for definitely defining the role of mutational analysis for patients stratification, dose selection and treatment duration in the adjuvant setting, are difficult because the rarity of disease, retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting are necessary.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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

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Write as mean ± SD or mean ± SE.

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