ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2013 August 28; 19(32): 5207-5394

Baishideng Publishing Group
www.wjgnet.com

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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World Journal of Gastroenterology

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Weekly Volume 19 Number 32 August 28, 2013

World Journal of Gastroenterology
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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1352 experts in gastroenterology and hepatology from 64 countries.

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World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2012 Impact Factor: 2.547 (34/74); Total Cites: 19145 (6/74); Current Articles: 944 (1/74); and Eigenfactor® Score: 0.06035 (6/74).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
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http://www.wjgnet.com

PUBLICATION DATE
August 28, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5207

World J Gastroenterol 2013 August 28; 19(32): 5207-5211
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Bile duct cyst in adults: Interventional treatment, resection, or transplantation?

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Received: May 26, 2013 Revised: July 15, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

Cystic dilatations of the bile ducts may be found along the extrahepatic biliary tree, within the liver, or in both of these locations simultaneously. Presentation in adults is often associated with complications. The therapeutic possibilities have changed considerably over the last few decades. If possible, complete resection of the cyst(s) can cure the symptoms and avoid the risk of malignancy. According to the type of bile duct cyst, surgical procedures include the Roux-en-Y hepaticojejunostomy and variable types of hepatic resection. However, the diffuse forms of Todani type Ⅴ cysts (Caroli disease and Caroli syndrome) in particular remain a therapeutic problem, and liver transplantation has become an important option. The mainstay of interventional treatment for Todani type Ⅲ bile duct cysts is *via* endoscopic retrograde cholangiopancreatography. The diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and liver transplantation all have their place in the treatment of this heterogeneous disease group. They should not be seen as competitive treatment modalities, but as complementary options. Each patient should receive individualized treatment after all of the clinical findings have been considered by an interdisciplinary team.

Key words: Bile duct cyst; Caroli syndrome; Caroli disease; Hepatic resection; Liver transplantation; Interventional treatment

Core tip: This is an invited editorial on the role of different treatment options for patients with bile duct cysts. It is not meant to be a thorough review on the numerous aspects of this disease, but instead intends to provide critical insights into current developments in interventional and surgical therapies, defining their potential and indicating that they should not be seen as competitive but as complementary options. The diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and liver transplantation all have their place in the treatment of this heterogeneous disease group.

Cerwenka H. Bile duct cyst in adults: Interventional treatment, resection, or transplantation? *World J Gastroenterol* 2013; 19(32): 5207-5211 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5207.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5207

INTRODUCTION

The modified Todani[1,2] classification that is widely used for bile duct cysts has several drawbacks. In particular, it combines different disease processes and does not account for the risk of malignant transformation or differences in epidemiology, pathogenesis, complications, and treatment. As a result, its clinical significance has been the subject of critical discussion and further modifications have been proposed. Moreover, there is uncertainty on the categorization of some variants.

Type I bile duct cysts comprise the subtypes I A (cystic dilatation of the common bile duct), I B (segmental dilatation of the common bile duct), and I C (fusiform dilatation extending to the common hepatic duct). Type

Ⅱ is a true diverticulum in the extrahepatic duct (supraduodenal). Type Ⅲ is a choledochocele confined to the common bile duct within the duodenal wall. An important differential diagnosis for a type Ⅲ cyst is a juxtapapillary duodenal diverticulum. Type Ⅳ is divided into type Ⅳa (multiple intra- and extrahepatic bile duct cysts) and type Ⅳb (multiple extrahepatic bile duct cysts, which are less common than type Ⅳa). Finally, type Ⅴ corresponds to Caroli disease (single or multiple intrahepatic bile duct cysts). When associated with congenital hepatic fibrosis, type Ⅴ is termed Caroli syndrome and is inherited as an autosomal recessive trait[3-5].

Caroli disease (CD) and Caroli syndrome (CS) are part of a broader spectrum of diseases with ductal plate malformations. These diseases have a close relationship with congenital kidney disorders, notably autosomal recessive polycystic kidney disease[6]. Generally, Caroli syndrome is characterized by early onset, with rapid disease progression due to the combination of cholangitis and portal hypertension[7-9]. Ductal plate malformations affect different levels of the intrahepatic biliary tree, including the large and proximal ducts in CD, the smaller ducts in CS and congenital hepatic fibrosis, and the more peripheral interlobular ducts in polycystic liver disease and von Meyenburg complexes[10,11]. Type Ⅴ cysts can be considered as a distinct disease entity from types I-Ⅳ, and type Ⅲ might be an anatomical variation rather than a true dilatation of the common bile duct[12]. Indeed, Ziegler *et al*[13] suggested that the classification of bile duct cysts should not include choledochoceles (type Ⅲ), as they differ from choledochal cysts with respect to age, gender, presentation, pancreatic duct anatomy, and their management.

Michaelides *et al*[14] described a dilatation of the central portion of the cystic duct apart from the dilatation of the common hepatic and common bile duct, giving the cyst a bicornal configuration. They suggested classifying this finding as a further subtype of Todani I cysts, namely Todani ID. However, Calvo-Ponce *et al*[15] have already proposed Todani ID as a cyst above the junction of the common hepatic duct and the cystic duct.

Okada *et al*[16] described a "common channel syndrome" and Lilly *et al*[17] used the term "forme fruste choledochal cyst" for a long common channel secondary to a proximal junction of the common bile duct and pancreatic duct with stenosis of the distal common bile duct, combined with cholecystitis and the classical pathological features of a choledochal cyst in the wall of the common bile duct. Forme fruste choledochal cysts are associated with only minimal or no dilatation of the extrahepatic bile duct, pancreaticobiliary maljunction, and protein plugs or debris in the common channel[18-20]. The cut-off diameter of the extrahepatic bile duct above which the diagnosis of a forme fruste is unacceptable has been arbitrarily described as 10 mm[20].

Kaneyama *et al*[21] reported variants with a type Ⅱ diverticulum arising from a type IC bile duct cyst, resulting in "mixed type I and Ⅱ" cysts. Visser *et al*[22] argued that all varieties of type I bile duct cysts have some element of intrahepatic dilatation. They concluded that type I and Ⅳa cysts only differ in terms of the extent of intrahepatic dilation, which makes discriminating between these two types rather arbitrary. Furthermore, an additional category, termed type Ⅵ, has been used for cystic malformations of the cystic duct[23,24]. However, these cysts could also be classified as a subtype of type Ⅱ[25]. Loke *et al*[26] described diverticular cysts of the cystic duct, whereas Wang *et al*[27] reported a patient with type I and type Ⅲ choledochal cysts that occurred simultaneously.

Notwithstanding these drawbacks, the crucial advantages of the Todani classification are its widespread use and reproducibility, which allow comparisons among the various published studies that have been built upon it. Therefore, its further use is to be advocated, despite its shortcomings. For this reason, the Todani system is also used in this editorial as a background for the therapeutic considerations.

INTERVENTIONAL TREATMENT

For type Ⅲ bile duct cysts, endoscopic retrograde cholangiopancreatography (ERCP) is an important diagnostic tool, and interventional therapy *via* ERCP is the mainstay of treatment. For the diagnosis of the other types, the less-invasive magnetic resonance cholangiopancreatography (MRCP) has widely replaced ERCP and represents the "gold standard" approach. However, ERCP remains the imaging modality of choice for type Ⅲ cysts because it also enables a simultaneous therapeutic sphincterotomy to be performed. Originally, type Ⅲ cysts were treated by transduodenal excision and sphincteroplasty; however, endoscopic sphincterotomy is now considered to be sufficient. Ohtsuka *et al*[28] reported malignancies in 3 out of 11 patients with type Ⅲ cysts. Therefore, endoscopic surveillance is recommended. Interventional therapy with ERC and shock-wave lithotripsy, in addition to antibiotics and bile acid treatment (ursodeoxycholic acid, UDCA)[29], is also used for type Ⅴ cysts, but cannot be expected to be curative. Its success is usually only temporary, and recurrent episodes of cholangitis cannot be prevented[30]. Internal biliary stents have also been described as a therapeutic option in anecdotal case reports[31,32].

A crucial argument against long-term treatment with interventional techniques and for removal of the cyst is the increased risk of malignancy. About 2.5%-17.5% of patients with bile duct cysts develop malignancies, and this incidence increases with age[33]. In a series of 38 adult patients published by Visser *et al*[22], 21% developed malignancies. The underlying mechanisms that cause carcinogenesis may be complex, as a consequence of chronic inflammation, cell regeneration, and DNA breaks leading to dysplasia. Pancreatic reflux might also play a role in causing K-ras mutations, cellular atypia, and the overexpression of p53[34]. Malignancy in Caroli disease has been reported to occur in 7%-15% of patients[33], underlining the need for surgery and surveillance.

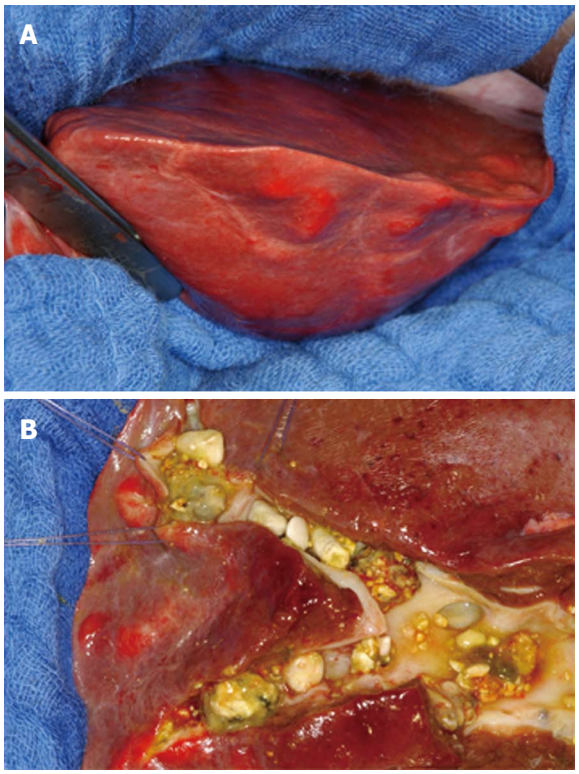

Figure 1 Intraoperative picture (A) and a detailed view (B) of the operative specimen (left hepatic lobe) in a patient with Todani type V bile duct cysts.

RESECTION

Complete resection of cysts and Roux-en-Y hepaticojejunostomy (RYHJ) is the procedure of choice for type I and IVb bile duct cysts. The success rate of this approach has been reported as 92%[35]. If adequate, the procedure may be performed laparoscopically. The benefits of the laparoscopic approach are also underlined in the reports by Gander *et al*[36] and by Palanivelu *et al*[37]. Potential complications of RYHJ include cholangitis, pancreatitis, biliary calculi, and, rarely, malignancy. Watanabe *et al*[38] reported that malignancy occurred in less than 1% of patients after a previous cyst excision, but higher incidences have been observed that may be due to incomplete cyst removal. Postoperative complications are usually seen in patients with inflammation and fibrosis at the time of surgery.

For forme fruste choledochal cysts, excision of the malformed ductal tissue with biliary reconstruction is required, as cholecystectomy alone is an inadequate treatment[39].

In all cases, complete removal of the cyst makes a decisive difference. With incomplete removal, Liu *et al*[40] reported a malignancy rate of 33.3%, compared to 6% after complete resection. Simple excision may be feasible for type II cysts, with ligation at the neck and without the need for biliary reconstruction. If possible, a laparoscopic approach may be advantageous[41].

For type IVa cysts, a tailored approach is necessary. Visser *et al*[22] suggested that excision of the extrahepatic component with hepaticojejunostomy should be performed; however, in cases with symptomatic intrahepatic affections (with stones, cholangitis, or biliary cirrhosis), treatment should correspond to that used for type V cysts, with hepatic resection for localized disease and transplantation for diffuse forms.

Although reported numbers in the literature are low, there is broad consensus that hepatic resection is the therapy of choice in patients with localized forms of type V cysts (Figure 1). If patients with monolobar disease remain asymptomatic, they will only require supportive dissolution therapy and surveillance; however, the risk of malignancy also has to be considered. For example, Kassahun *et al*[42] determined a cholangiocarcinoma incidence of 9.7%, whereas Ulrich *et al*[30] reported 9.1% and Bockhorn *et al*[43] reported 25%.

In symptomatic patients with acute cholangitis, a variable symptom-free interval may be achieved by using antibiotic treatment with or without endoscopic sphincterotomy and calculi removal or lithotripsy. However, most of these patients will develop recurrent cholangitis, chronic inflammation, and an increased risk of cholangiocarcinoma. These patients are best treated by liver resection if their hepatic function is preserved and there are no contraindications to liver surgery.

In earlier publications, a rather small proportion of monolobar disease (20%-25%) was described, with almost 90% of these cases located in the left hepatic lobe. Due to small patient numbers, the prevalence of localized CD may have been underestimated. Recent studies indicate higher percentages (80%) of patients with localized disease[30,42] and a more variable distribution of disease between the left and right lobes.

TRANSPLANTATION

Diffuse forms of type V cysts (CD and CS) remain a therapeutic problem. In patients with these cysts, combined procedures with partial hepatectomy and biliodigestive anastomoses[44,45] have been described, but transplantation offers the only curative option. The progression of congenital hepatic fibrosis in CS and the development of secondary biliary cirrhosis in patients with CD may also lead to portal hypertension that is not treatable by conservative means. De Kerckhove *et al*[46] reported congenital hepatic fibrosis in 27% of their patients and the primary indication for orthotopic liver transplantation was recurrent cholangitis (90%).

An important argument for liver transplantation is the avoidance of cholangiocarcinoma development[47]. Concerns include the choice of an appropriate time point for transplantation, procedural risks, and the use of immunosuppression in young and otherwise healthy individuals. Potential postoperative complications include rejection and vascular thrombosis[46]. Pre-transplant workup of these patients for occult cholangiocarcinoma is crucial.

In patients with associated polycystic kidneys and renal failure, immunosuppression after kidney transplantation may predispose to severe cholangitis, and therefore, combined liver/kidney transplantation should be considered. The results of liver transplantation for diffuse forms of type V cysts compare favorably with those of transplantations for other indications. In the review carried out by Millwala *et al*[48], the overall graft and patient survival rates at 1, 3, and 5 years were 79.9%, 72.4% and 72.4%, and 86.3%, 78.4% and 77%, respectively; living donor transplantation was performed in 3.8% of cases. In the single-center study by Habib *et al*[49], overall graft and patient survival rates at 1, 5, and, 10 years were reported as 73%, 62%, and 53%, and 76%, 65% and 56%, respectively.

In conclusion, the diagnostic term "bile duct cyst" comprises quite different pathological and clinical entities. Interventional therapy, hepatic resection, and transplantation all have their place in the treatment of this heterogeneous disease group. They should not be seen as competitive, but complementary options. In spite of several shortcomings, the modified Todani classification offers a basis for treatment planning and for comparing results reported in the literature. Each patient, however, should receive tailored individual treatment after all findings have been discussed by an interdisciplinary team.

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P- Reviewers Cucchetti A, Kim DG **S- Editor** Wen LL
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5212

World J Gastroenterol 2013 August 28; 19(32): 5212-5226
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

MicroRNAs and liver cancer associated with iron overload: Therapeutic targets unravelled

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Received: March 26, 2013 Revised: May 15, 2013
Accepted: May 18, 2013
Published online: August 28, 2013

Abstract

Primary liver cancer is a global disease that is on the increase. Hepatocellular carcinoma (HCC) accounts for most primary liver cancers and has a notably low survival rate, largely attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis. MicroRNAs (miRNAs/miRs) are regulatory RNAs that modulate protein synthesis. miRNAs are involved in several biological and pathological processes including the development and progression of HCC. Given the poor outcomes with current HCC treatments, miRNAs represent an important new target for therapeutic intervention. Several studies have demonstrated their role in HCC development and progression. While many risk factors underlie the development of HCC, one process commonly altered is iron homeostasis. Iron overload occurs in several liver diseases associated with the development of HCC including Hepatitis C infection and the importance of miRNAs in iron homeostasis and hepatic iron overload is well characterised. Aberrant miRNA expression in hepatic fibrosis and injury response have been reported, as have dysregulated miRNA expression patterns affecting cell cycle progression, evasion of apoptosis, invasion and metastasis. In 2009, miR-26a delivery was shown to prevent HCC progression, highlighting its therapeutic potential. Several studies have since investigated the clinical potential of other miRNAs with one drug, Miravirsen, currently in phase Ⅱ clinical trials. miRNAs also have potential as biomarkers for the diagnosis of HCC and to evaluate treatment efficacy. Ongoing studies and clinical trials suggest miRNA-based treatments and diagnostic methods will have novel clinical applications for HCC in the coming years, yielding improved HCC survival rates and patient outcomes.

Key words: MicroRNAs; Liver cancer; Iron regulation; Hepatitis C; Therapeutic targets

Core tip: Hepatocellular carcinoma (HCC) has a high incidence and low survival rate, largely attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis. MicroRNAs (miRNAs) are regulatory RNAs that modulate protein synthesis and are involved in several biological and pathological processes including the development and progression of HCC. miRNAs represent important new targets for therapeutic intervention for HCC and have potential as diagnostic and prognostic HCC biomarkers. Ongoing studies and clinical trials suggest miRNA-based treatments and diagnostic methods will have clinical applications for HCC in the coming years, yielding improved HCC survival rates and patient outcomes.

Greene CM, Varley RB, Lawless MW. MicroRNAs and liver cancer associated with iron overload: Therapeutic targets unravelled. *World J Gastroenterol* 2013; 19(32): 5212-5226 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5212.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5212

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 85%-90% of primary liver cancers; it ranks as the fifth most common cancer worldwide and the third leading cause of death from malignancy[1]. The development and progression of HCC is a multistage process, with transformation typically beginning in hepatocytes of livers undergoing chronic hepatitis or cirrhosis[2]. The major risk factor for HCC is chronic hepatitis due to infection with the hepatitis B or hepatitis C virus (HBV/HCV) accounting for 80%-90% of all HCC cases worldwide[3]. The other most important risk factors for hepatocarcinogenesis are alcoholic and non-alcoholic steatohepatitis-associated cirrhosis; less common risk factors include genetic conditions such as hereditary haemochromatosis (HH), alpha-1 antitrypsin deficiency[4,5] and aflatoxin B1 intake. Regardless of the underlying risk factor, hepatocytes progress through several hyperplastic and dysplastic stages before eventually acquiring a malignant phenotype, with subsequent intrahepatic metastasis and distant spread of HCC cells[6]. The 5-year survival rate of patients with HCC remains quite low, between 6%-11%. This is attributable to late diagnosis, resistance to treatment, tumour recurrence and metastasis[2].

Previously, studies investigating HCC development and progression have focused on the therapeutic potential of targeting various genes and proteins[7]. However, a new group of regulatory RNA molecules has more recently been identified, called microRNAs (miRNAs). Involvement of miRNAs in HCC development and progression has been demonstrated; as such miRNAs have considerable diagnostic and therapeutic potential for HCC. Here, the role of miRNAs in the pathogenesis of HCC is reviewed with a focus on their regulation of iron homeostasis and in the setting of iron overload, a common pathological event observed in several liver diseases associated with HCC development. The relevance of miRNAs to HCC progression with regard to hepatic fibrosis and response to injury, as well as their contribution to cell cycle progression, evasion of apoptosis and metastasis is explored. Finally, the potential diagnostic and therapeutic value of miRNAs in HCC is discussed.

miRNAs

miRNAs are endogenous single stranded RNAs, approximately 22 nucleotides in length. They are non-coding but are important post-transcriptional regulators of gene expression. miRNAs were first discovered in 1993, and since then the considerable extent of the gene regulatory capacity of miRNAs has been investigated. These investigations have demonstrated that specific miRNAs have central roles in critical biological processes such as development, cell proliferation, apoptosis and oncogenesis. The mechanisms of action and biogenesis of miRNAs have been reviewed in detail[8,9].

Mature miRNAs enter the RNA-induced silencing complex (RISC) in the cytosol. In this complex miRNA can post-transcriptionally regulate gene expression. Their mechanism of action is determined by the level of complementarity between the miRNA and the 3'-untranslated region (UTR) target on the mRNA. In perfect complementarity, miRNA-mRNA binding induces mRNA cleavage and degradation by RISC. In imperfect complementarity, miRNA-mRNA binding represses target mRNA translation[10]. Occassionally, miRNAs can upregulate translation even in conditions of growth arrest[11]. However translation is more commonly inhibited and the target mRNAs are eventually degraded in cytoplasmic processing bodies[12].

Functional target sites on mRNAs usually consist of a 6-8-nt long sequence complementary to the miRNA sequence (followed by an adenosine), this is termed the miRNA "seed" sequence and is located at the 5' end of the miRNA[13]. The complementary sequence commonly referred to as a miRNA recognition element (MRE) is usually located in the 3'-UTR of the target mRNA. Some recent studies have shown miRNAs can also bind to MREs located in the 5'-UTR or the open reading frame[14-17]. Unusually miRNAs can act as decoys and bind to ribonucleoproteins independent of a seed sequence and RISC, thus interfering with roles requiring mRNA binding[18].

Given the considerable potential for variety in miRNA-mRNA interaction, it is not surprising that a single miRNA can target several genes[19-22]. In addition, approximately 60% of mRNAs carry at least one evolutionarily conserved MRE. Bioinformatic analysis predicts that the 3'-UTR of a single transcript is often targeted by several miRNAs, a prediction that has been validated experimentally for many genes[22]. The complex, widespread and cooperative regulation of gene expression by miRNAs is an important consideration when studying normal and pathological processes in terms of understanding the processes themselves and identifying potential biomarkers. Recently investigators have begun to study the role of miRNAs in the pathogenesis of HCC. In particular, several studies have demonstrated a role for miRNAs in HCC development and progression, wherein the importance of miRNAs in iron homeostasis and hepatic iron overload were highlighted.

Many risk factors underlie the development of HCC and one process commonly altered is iron homeostasis. Iron overload in the liver occurs in several liver diseases associated with the development of HCC, including chronic hepatitis due to HCV infection and also due to genetic conditions such as HH. Hepatic iron overload is an independent risk factor for the development of HCC[23] and emerging evidence points towards miRNAs as central regulators of iron homeostasis

miRNAs, HCC AND IRON OVERLOAD

Hepatic iron overload and HCC

Hepatocytes act as the principal site of iron storage in the body, storing iron as ferric oxyhydroxyapatite in the core of ferritin. During iron overload, the ability of hepatocytes to safely sequester iron is exceeded, denaturation of

ferritin subunits occurs leading to ionic iron release into the hepatocyte cytoplasm[24]. The effects of hepatic iron overload have been particularly well studied in patients with the inherited iron metabolism disorder, HH and in Africans with dietary iron overload.

Patients with HH, without timely appropriate treatment, almost always develop hepatic fibrosis and cirrhosis due to hepatic iron accumulation[25]. Similarly patients with African dietary iron overload can develop cirrhosis, albeit less often[26,27]. HCC is a potential complication in untreated HH patients associated with premature death[28,29]. Comparison studies have showed that cirrhosis plays a role in the development of HCC in HH[30,31] however, HCC can also develop in HH patients without cirrhosis, albeit rarely[32-37]. Together this suggests that hepatic iron storage could directly contribute to HCC development[38,39], in addition to its indirect effect as a cause of cirrhosis. This concept is in keeping with a study comparing cirrhosis incidence in HH and non-iron related liver diseases, where the risk of HCC was greater in HH[40]. Interestingly, despite HCC initially being thought not to occur in dietary iron overload, three case/control studies have demonstrated a causal association between African dietary iron overload and HCC, even after allowing for the confounding effects of cirrhosis, chronic HBV and HCV infection and prolonged aflatoxin B1 exposure[41-43]. Dietary iron overload resulting in HCC has also been reported in animal models[44,45] supporting the directly hepatocarcinogenic effects of hepatic iron accumulation.

HCC can also develop with other causes of hepatic iron accumulation namely, thalassaemia major, sideroblastic anaemia and hereditary spherocytosis[46-48]. Lesser degrees of hepatic iron accumulation are seen in other liver diseases, such as chronic HCV hepatitis and alcoholic liver disease. Nonetheless, it is thought to have an important role in these diseases[24]. One area of recent interest is hepatic iron accumulation with HCV infection. As the main risk factor for HCC development, HCV is particularly relevant to HCC. Iron promotes the initiation of HCV translation by increasing expression of eukaryotic initiation factor 3a and La protein, whereas inhibiting expression of these proteins suppresses HCV translation[49,50]. Interestingly the expression of the chief iron regulatory hormone, hepcidin, is suppressed in chronic HCV infected patients. Given that hepcidin expression has direct anti-viral activity against HCV in cell culture[51] this represents an exciting area of ongoing research.

Hepatic iron accumulation has also been implicated in non-alcoholic fatty liver disease (NAFLD). Hyperferritinemia is associated with higher hepatic iron and fat content in NAFLD[52], and is also an independent predictor of liver damage in NAFLD patients[53]. As altered iron trafficking is frequent in patients with NAFLD, one recent study investigated the role of the Ala736Val polymorphism of TMPESS6 (an inhibitor of hepcidin expression) in NALFD-associated hepatic iron accumulation[54]. Homozygosity for this polymorphism was associated with low hepatic iron stores and was negatively associated with hepatic iron accumulation independent of age, gender, human haemochromatosis (HFE) genotype and beta thalassaemia trait.

Pathogenesis of HCC in hepatic iron overload

A recent animal study examined the long-term effects of iron overload in HCC[44]. A high-iron diet was given to Wistar albino rats over 16 mo to induce hepatic iron overload. Altered hepatic foci developed in many animals by 20 mo. By 28 mo, these foci were more numerous and had become identical to the iron-free preneoplastic nodules seen in HH patients who develop HCC[55]. HCC was evident at 32 mo in the absence of portal fibrosis or cirrhosis. The mechanisms by which free iron induces hepatocarcinogenesis are not yet fully characterised but are likely due to the generation of reactive oxygen intermediates (ROI) and oxidative stress which damages DNA, lipids, and proteins resulting in both necrosis and apoptosis within hepatocytes[56-60]. Oxidative DNA damage correlates with cell immortalisation in HCC through induction of telomerase activity. This process has been associated with miR-92 over expression, a miRNA affecting specific cell proliferation and apoptosis pathways[61]. Iron overload leading to lipid peroxidation is also thought to contribute to HCC development[62-66]. Moreover, excess hepatic iron may induce immunologic alterations, leading to impaired immune surveillance of malignant transformation. Nontransferrin-bound iron can markedly suppress lymphocyte proliferation[67]. The same study showed that ferritin can inhibit lymphocyte proliferation. Indeed, the presence of both iron and ferritin were found to significantly reduce the tumouricidal function of macrophages[68]. In addition to its solitary effects, iron overload can act in tandem with other HCC risk factors to produce hepatocarcinogenesis. For example, dietary iron overload and aflatoxin B1 exposure have superadditive effects on mutagenesis rates[69]. Furthermore ROI generation and mutagenesis are synergistically increased in animal models with both risk factors, leading to greater DNA damage[70-73].

Control of cellular iron uptake by miRNAs: Most cells obtain iron from plasma *via* iron-bound transferrin (Tf-Fe2) uptake. Tf-Fe2 binds to TfR1 on the cell surface and the complex is internalised by clathrin-dependent endocytosis. Acidification of early endosomes aids iron release from transferrin[74], so that it can be reduced to Fe^{2+} by metalloreductases[75]. Transport into the cytoplasm occurs *via* endosomally-expressed Divalent metal transporter 1 (DMT1). Cell surface TfR1 levels reflect cellular iron requirements, with regulation of TfR1 expression mainly achieved by the IRE/IRP regulatory system[76]. However, recent studies have shown that the transferrin cycle is also controlled by miRNAs, at two separate steps (Figure 1A).

Cancerous cells have elevated TfR1 expression to meet the increased iron requirements of rapid cellular proliferation[77,78]. Conversely, differentiation of a human

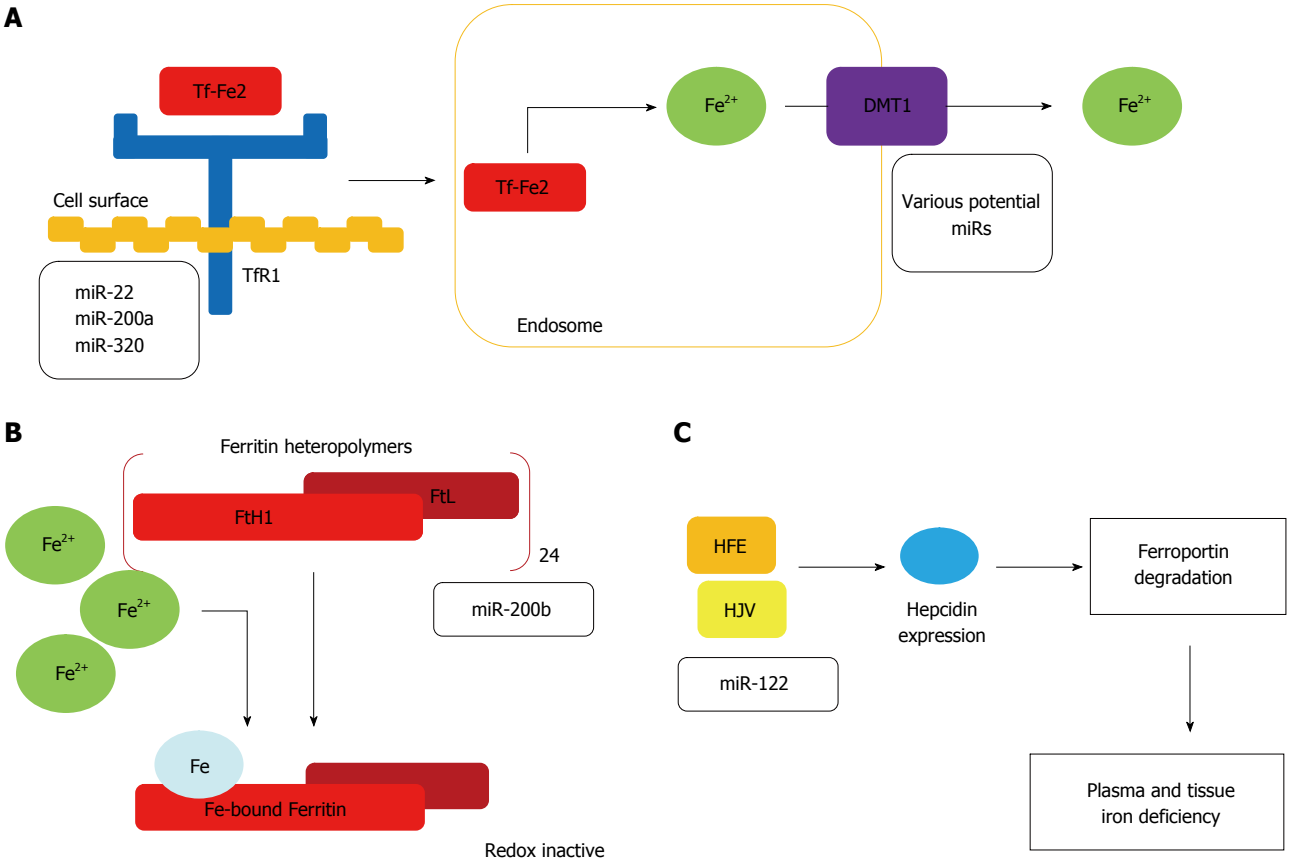

Figure 1 Effect of microRNAs on iron uptake, storage, and systemic regulation. A: Iron-bound transferrin (Tf-Fe2) binds to the transferrin receptor TfR1 which is regulated by microRNA (miR)-22, miR-200a and miR-320. The complex is endocytosed leading to release of iron, its reduction to Fe^{2+} and transport to the cytoplasm *via* DMT1 which may be regulated by various miRNAs; B: miR-200b regulates ferritin heavy (FtH1) and light (FtL) chains. Ferritin polymers containing 24 subunits detoxify excess iron *via* FtH1's ferroxidase activity and store intracellular iron; C: Levels of human haemochromatosis (HFE) protein and hemojuvelin (HJV) are regulated by miR-122, the levels of which are decreased in hereditary haemochromatosis. Reciprocal increases in HFE and HJV, in turn, enhance expression of hepcidin leading to decreased iron absorption due to degradation of ferroportin. DMT: Divalent metal transporter.

leukaemia cell line decreases TfR1 expression[79]; this is accompanied by reciprocal increases in miRNAs predicted to bind to the TfR1 3'-UTR (miR-22, miR-200a and miR-320). Of these, miR-320 was demonstrated to suppress the activity of a luciferase reporter vector under the control of the TfR1 3'-UTR[80]. Similarly, enforced miR-320 expression in a lung carcinoma cell line can reduce TfR1 expression and slow cell cycle progression and cell growth. This growth inhibitory effect can be reversed by treatment with a soluble iron solution suggesting that reduced TfR1 expression in miR-320-overexpressing cells lowers iron availability and reduces cell proliferation[81]. Currently, it is unknown whether miR-320-mediated TfR1 regulation is limited to cancer cells or whether it has a role under normal physiological conditions.

In addition to miRNA-dependent TfR1 regulation, miRNAs control the transferrin cycle at the release of iron from the endosome *via* DMT1. The gene coding for DMT1 (*SLC11A2*) produces four variant mRNA transcripts. These differ either at their 5' end due to alternative promoter usage (DMT1A and 1B isoforms), or at the 3' end, due to alternative splicing determining the presence or absence of an IRE sequence motif[82]; only the IRE-containing isoforms are controlled in response to cellular iron levels by IRP binding[83]. All DMT1 isoforms can transport iron and, with the exception of the duodenal 1A isoform, are ubiquitously expressed[84]. Of note, miRNA-controlled DMT1 expression by let-7d can contribute to the uptake of non-transferrin bound iron[85]. Further studies are needed to determine how miRNA-dependent control of DMT-1 expression is integrated with additional DMT-1 control mechanisms.

Importantly, as miRNA maturation requires iron in the form of heme[86], the finding that miRNAs control cellular iron uptake suggests a possible regulatory loop in which iron is needed for the efficient synthesis of mature miRNAs, while certain mature miRNAs control cellular iron uptake.

Control of cellular iron storage by miR-200b: Ferritin heteropolymers consist of 24 subunits of heavy (FtH1) and light (FtL) chains that bind iron from the cytoplasmic "labile iron pool"[87]. The FtH1 subunit has ferroxidase activity necessary for iron deposition in ferritin. Ferritin detoxifies excess iron into a redox-inactive form, preventing chronic oxidative stress and subsequent cell and

tissue damage. Ferritin also acts as an intracellular iron store mobilised *via* proteasomal and lysosomal degradation. One recent study showed that human breast cancer cells with an aggressive mesenchymal phenotype express significantly higher FtH1 and FtL mRNA and protein levels and have a smaller labile iron pool compared to breast cancer cells with a less aggressive epithelial phenotype[88]. High FtH1 concentrations correlated with low miR-200b expression, a miRNA that binds both FtH1 and FtL 3'UTRs (Figure 1B). Of clinical relevance, miR-200b transfection improved sensitivity of breast cancer cells to doxorubicin. Additionally, patients with higher plasma ferritin levels showed worse treatment outcomes, emphasising the clinical significance of this facet of iron regulation. These findings suggest that down regulation of miR-200b in human breast cancer contributes to increased cancer aggressiveness. Whether FtH1 and FtL are regulated by miR-200b in hepatocytes and if this has implications for HCC remains to be determined[89,90].

Control of systemic iron regulation by miR-122: The liver regulates systemic iron homeostasis *via* hepcidin and monitors systemic iron availability through genes involved in HH (*e.g.*, HFE, hemojuvelin and TfR2), the bone morphogenetic protein (Bmp) 6 and the Smad4 protein. These all function in the regulation of hepcidin transcription. Low hepcidin activity due to mutations in HFE, hemojuvelin, TfR2 or hepcidin itself lead to the development of HH which is associated with increased iron uptake from the diet and increased iron release from macrophages.

miR-122 is selectively expressed in the liver. One recent study demonstrated that miR-122 expression is reduced in a mouse model of HFE-mutated HH[91]. Depletion of miR-122 in wild type mice led to low systemic iron levels, decreased plasma iron levels and lower transferrin iron binding capacity. These events in turn resulted in an insufficient iron supply to erythroid cells and a mild impairment of haematopoiesis[91]. Furthermore, the iron contents of the liver and spleen were also reduced. Interestingly, miR-122 depletion altered systemic iron homeostasis through changes in the level of expression of genes involved in the sensing of systemic iron levels (*i.e.*, HFE, Hemojuvelin, and Bmpr1a), as well as genes that transmit signals *via* the Bmp/Smad signalling pathway, to regulate hepcidin transcription[91]. This study also validated HFE and hemojuvelin as direct targets of miR-122 (Figure 1C).

This suggests a miR-122-dependent regulatory loop that controls systemic iron homeostasis whereby depletion of miR-122 derepressed HFE and hemojuvelin expression, in turn increasing hepcidin transcription. As a result, high circulating hepcidin levels can enhance the degradation of ferroportin on target cells, leading to lower iron absorption from the diet and iron release from macrophages. This likely leads to plasma and tissue iron deficiency, with mild impairment of erythropoiesis. miR-122 levels are not regulated as a result of iron accumulation in the liver of HH patients, but more likely as a consequence of the signalling activities reduced by a lack of HFE which is known to attenuate BMP/Smad signalling in HH patients and its respective murine disease model[92].

The finding that miR-122 regulates systemic iron homeostasis is one of a growing number of functions known for this liver-specific miRNA. For example, miR-122 is necessary for HCV infection and replication, as well as for responsiveness to interferon therapy[93-95], all processes involving alterations in iron homeostasis[96]. miR-122 levels are reduced in cirrhosis[97] and HCC[98,99], two pathologies known to be exacerbated by increased liver iron levels[24]. Evidently, miRNAs have an important role in the maintenance of iron homeostasis, given their roles in controlling the level of cellular uptake of iron-bound transferrin, iron storage by ferritin, and hepatic control of systemic iron levels *via* hepcidin (Figure 1). Furthermore, tissue iron overload causes oxidative stress that itself has been shown to alter miRNA expression[100,101].

Overall, these findings suggest that miRNAs control large regulatory networks that link microenvironmental stress, such as oxidative stress and hypoxia to the regulation of iron metabolism. As the maintenance of iron homeostasis is critical for many essential cellular functions, it is expected that several more miRNAs that directly or indirectly control iron-related genes will be discovered. Given the role of miRNAs in regulating iron homeostasis and the significance of iron overload to the development of HCC, miRNAs likely play an important role in the pathogenesis of HCC (Table 1). However, further studies elucidating the full extent of miRNAs' functions in iron homeostasis under normal conditions are needed to improve our understanding of the role of miRNAs in pathologies such as HCC.

miRNAs AND HCC PROGRESSION

In HCC miRNAs can act as oncogenes, promoting hepatocyte progression to HCC, or as tumour suppressors, preventing this process[2]. Increased oncogenic miRNA levels result in reduced translation of their gene targets, contributing to HCC development and progression. By contrast, miRNAs acting as tumour suppressors prevent the expression of their oncogenic targets and hence the downregulation of such miRNAs permits greater expression of these oncogenic genes, again contributing to HCC development and progression. Progression from normal hepatocytes to HCC is a multistage process. Several changes in the liver structure and in normal cell processes must occur for this progression to continue, mediated in part by altered miRNA expression profiles. These include liver fibrosis and hepatic stellate cell-mediated liver regeneration, while at the molecular level changes in cell cycle progression, susceptibility to apoptosis and capacity for invasion and metastasis are needed.

Liver fibrosis, hepatic stellate cells and liver regeneration

miRNA expression profiles show considerable overlap

Table 1 MicroRNAs with a role in hepatocellular carcinoma

miRNAs	Function	Outcomes
miR-22 miR-200a miR-320	Predicted to bind iron-bound transferrin receptor (TfR1)	Targets TfR1, DMT1 expression thereby inhibiting cell cycle progression and growth
miR-200b	Targets ferritin heteropolymers (FtH1, FtL)	Decreased miR-200b linked with enhanced cancer aggressiveness *via* increased iron indices
miR-122	Targets HFE, hemojuvelin, BMPr1a, BMP/SMAD signalling, hepcidin	Control of systemic iron homeostasis. Decreased miR-122 corresponds to decreased HFE and hemojuvelin expression. This correlates with increased hepcidin expression

HCC: Hepatocellular carcinoma; miR/miRNA: MicroRNA; DMT: Divalent metal transporter; HFE: Human haemochromatosis; BMP: Bone morphogenetic protein; FtH: Ferritin heavy; FtL: Ferritin light.

in fibrotic disorders. The most significant mediators are the miR-29 family, important in regulating translation of extracellular matrix components and effectors of cellular differentiation[102]. Also important are miRs affecting translation of proteins involved in the pro-fibrotic transforming growth factor (TGF)-β/SMAD signalling pathway. Microarray analyses in a CCl_4 rodent model of hepatic fibrosis have shown 31 differentially expressed miRs, 10 of which are over expressed in fibrotic tissue including miR-125-p, -199b, -221 and -302c[103]. This same study revealed a significant down regulation in 21 miRs, most notably the miR-29 family. Down regulation of miR-29b and miR-29c was independently confirmed in a bile duct ligation model and similar observations for miRs-29a/b/c have been reported in humans liver tissue samples of patients with a Desmet fibrosis score of 2-4[104].

Hepatic fibrosis is also affected by miR-132 levels. In two different models of hepatic fibrosis (BDL and CCl_4), where a significant reduction in miR-132 levels was observed, this down regulation was found to alter the activity of hepatic stellate cells (HSCs). HSCs are the main effector cells of hepatic fibrosis, acting as the primary source for type I collagen deposition following liver injury. HSC activation occurs in response to hepatic insults including viral infection, alcohol consumption and obesity. During their activation, quiescent lipid-rich cells are transdifferentiated into fully activated myofibroblasts. The activated cells can secrete pro-fibrogenic mediators such as TGF-β, and produce extracellular matrix components[105]. Involvement of miRNAs in the process of HSC activation has been demonstrated. For example, let-7 family members are significantly up regulated in HSCs of BDL animals whereas miR-150, -187, -194 and -207 are down regulated[106]: over expression of miR-150 and miR-194 in human HSCs can inhibit HSC proliferation and prevent HSC transdifferentiation[106]. miR-150 together with another miR, miR-94, inhibits c-Myb and Rac-1, two proteins involved in pathways contributing to hepatic fibrosis development and progression. Further studies investigating differential miRNA expression in quiescent and activated rat HSCs showed that miR-15b and miR-16 are also implicated in HSC activation[107,108]. This process is also regulated by miR-27a and b which are up regulated and in turn repress RXRα[109]. Of interest miR-132 activates the methylCpG binding protein MeCP2 and components of the polycomb repressive process. Down regulation of miR-132, as seen in hepatic fibrosis, permits MeCP2 translation. This protein is subsequently recruited to the 5'UTR of PPARγ mRNA and through alteration of methylation patterns suppresses the quiescent profile of HSCs[110] - this is an example of a miRNA acting as an activator rather than an inhibitor of gene expression. Thus as our understanding of the role of miRNAs in the regulation of HSC differentiation improves so will the understanding of liver pathology and hepatic responsiveness to injury.

Cell cycle progression

Aberrant cell cycle control is necessary for the development and progression of all human cancers, including HCC. Cell cycle regulation by oncoproteins and tumour suppressors is often defective resulting in increased cell proliferation. miRNAs targeting the main proliferation pathways have been identified in HCC. These miRNAs exert their effects through an interaction with essential regulators of the cell cycle, including cyclin-dependent kinase enzyme (CDK) complexes, Cip/Kip family proteins which act as cell cycle inhibitors, and the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, among others.

Cyclins are positive cell cycle regulators, controlling cell cycle stage advancement *via* activation of CDKs. Cyclin D2 and E2, mediators of cell cycle arrest, are directly targeted by miR-26a; low miR-26a levels are frequently found in HCC[111]. Modulation of cyclin G1 affects transcriptional activity and p53 protein stability, resulting in reduced G2-M phase and lower invasive capacity of HCC cells[112]. miR-122 inhibits hepatocyte growth by targeting cyclin G1 expression, however it is barely detectable in primary human HCC[113]. Levels of miR-122 are determined by several key regulatory molecules, including the transcription factors HNF1A, HNF3A and HNF3B[114]. Low miR-122 correlates with high serum response factor, a validated miR-122 target and important promoter of tumour development[115]. Expression of miR-195 is also reduced in HCC. Normally it regulates expression of cyclin D1, CDK6 and EnF3 however in its absence there is a failure to induce cell cycle arrest at the G1-S checkpoint[116]. CDK6 is also targeted by miR-124, a miRNA which blocks G1-S transition. miR-124 is silenced in HCC by CpG methylation, as is miR-203[117].

Another method by which oncogenic miRNAs contribute to cell cycle progression is *via* inhibition of cyclin-dependent kinase inhibitors (CDKIs), most notably the members of the Cip/Kip family. Both miR-106b and miR-93 are overexpressed in HCC and directly target

p21 and promote cell cycle progression[118]. miR-221 and miR-222 both inhibit expression of p27 mRNA, another member of the Cip/Kip family[119] whilst miR-221 also regulates the CDKI p57[120]. Direct targeting of these two CDKIs leads to greater numbers of HCC cells in the S-phase thus promoting cell growth.

PI3K has an important role in balancing cell survival and apoptosis. Its activation leads to increased cell growth *via* phosphorylation of mTOR by AKT kinase, an effect that is inhibited by PTEN. mTOR is a target of miR-199a-3p; restoring normal levels of miR-199a-3p can cause cell cycle arrest in HCC by blocking the G1-S transition, sensitising cells to doxorubicin[121]. miR-221 and miR-222, in addition to their effect on p27 also target DNA damage-inducible transcription factor 4 (DDIT4), a modulator of mTOR signalling[122]. PTEN is directly targeted by miR-21, -221 and -222; all three are often found to be overexpressed in HCC[123,124]. As such, suppression of PTEN resulting in increased PI3K/AKT pathway activation is an important mediator of HCC cell survival.

Other important cell cycle regulators are known targets of aberrantly-expressed miRNAs in HCC. Let-7g down regulates c-Myc, an oncogenic transcription factor. This suppresses HCC cell proliferation through reduced c-Myc-induced miR-17-92 transcription, a tumour-promoting miR[125,126]. Others such as miR-1[127] and miR-375[128] suppress HCC cell proliferation whereas miR-18a stimulates proliferation *via* targeting the ESR1 gene thereby preventing oestrogen's protective effects against HCC in females[129].

These studies emphasise the important role that miRNAs have in the progression of HCC by regulating oncogenes and tumour suppressors, and a number of miRNAs have now been identified in this context.

miRNAs: INVASION, METASTASIS AND APOPTOSIS

Evasion of apoptosis

Evasion of apoptosis is another key step in malignant transformation and tumour progression. This allows cells to escape normal surveillance mechanisms, enabling continued survival in the tumour microenvironment. The tumour suppressor gene *p53* increases miR-34 expression leading to cell cycle arrest and apoptosis, whereas low miR-34 levels, as are frequently seen in HCC, are believed to contribute to apoptosis evasion[130-133]. miRNAs directly target the Bcl-2 family of genes, their proteins being either pro-apoptotic (Bim, Bmf, Bax, Bak, Bid) or anti-apoptotic (Bcl-2, Bcl-W, Bcl-XL, Mcl-1)[134]. miR-122 and let-7b regulate Bcl-w and Bcl-XL, respectively, whilst Mcl-1 is regulated by miR-101 and miR-29[104,135-137]. Reduced levels of all of these miRNAs are often seen in HCC thus increasing resistance to apoptosis. Bcl-2 is also targeted by miR-29[104]; increasing miR-29 levels can sensitise HCC cells to pro-apoptotic signals, a finding of great therapeutic application potential. With respect to miRNA regulation of pro-apoptotic Bcl-2 family members, miR-221 and miR-25 are commonly over expressed in HCC and target Bmf and Bim, respectively[138,139]. miRNAs can also target other apoptosis-related genes. miR-602 is increased in HBV-related HCC, it targets RASSF1A to exert an anti-apoptotic effect[140].

Invasion and metastasis

Invasion and metastasis are two hallmarks of cancers and the leading causes of cancer-related mortality. Survival rates after curative resection of HCC are still poor due to high recurrence secondary to intrahepatic metastasis. Given this, a better understanding of the mechanisms underlying invasion and metastasis is critical to improvements in patient survival. Several metastasis-related genes important in HCC have been identified, and with them, several miRNAs promoting and preventing metastasis in HCC.

miRNAs promoting metastasis: As mentioned, levels of miR-21, -221 and -222 are increased in HCC[124]. These miRNAs directly target PTEN, contributing to cell growth but also mediating cell invasion. miR-221 and miR-222 also modulate the expression of TIMP3 and phosphatase 2A subunit B (PPP2R2A), thereby preventing inactivation of metalloproteases, important enzymes involved in cell migration and invasion, and activating the PI3K pathway[124,141]. miR-181b is induced by TGF-β and also targets TIMP3 on a functional level, increasing MMP2 and MMP9 activity[142]. The TGF-β-mediated metastasis pathway is well characterised, and this TGF-β/miR-181/TIMP3 axis may be an important component. One study has also shown a novel miRNA, miR-143 is induced by NFκB, promoting metastasis of HBV-related HCC by inhibiting expression of fibronectin[143]. High miR-17-5p levels are often found in HCC. This miRNA activates p38 mitogen-activated protein kinase and leads to greater heat shock protein 27 phosphorylation thereby promoting HCC invasion[144].

The chromosomal region 8q24 is implicated in metastasis in HCC. Two frequently amplified miRNAs contained within, miRNA-30d and miRNA-151, are involved in HCC invasion and metastasis[145,146]. An increased miR-30d expression is frequently seen in HCC enhancing metastasis through repression of G-αi2. This can contribute to metastasis both within the liver and to the lung. RhoGDIA, thought to be a suppressor of HCC metastasis is targeted by miR-151; with subsequent activation of Rac1, Cdc42 and Rho GTPases enhancing cell migration and invasion[134]. Moreover, this miRNA is often co-expressed with host gene focal adhesion kinase (FAK); it can function synergistically with FAK to increase HCC cell motility and spread[134].

miRNAs preventing metastasis: ADAM10 (a distintegrin and metalloprotease family 10), serum response factor (SRF), and insulin-like growth factor 1 receptor (Igf1R) promote tumorigenesis. These are validated targets of

Table 2 MicroRNAs as potential diagnostics and therapeutics for hepatocellular carcinoma

miRNAs	Detail	Relevance
miR-221	4.8 fold higher in HCC patients, positively correlates with cirrhosis, tumour size and stage. Negatively correlates with overall survival	Potential circulating biomarker
miR-199a	Reduced and significantly associated with HCC	Potential circulating biomarker
miR-16	Reduced and significantly associated with HCC	Potential circulating biomarker
miR-26	Low levels associated with high IL-6 and shorter survival	Potential biomarker to assess prognosis of HCC
miR-375	Lower than normal levels associated with β-catenin mutation	Potential for HCC classification system, determine treatment allocation
miR-107	Reduced levels associated with HFN 1α	Potential for HCC classification system, use to determine treatment allocation
miR-122	Expression inhibited using Miravirsen LNA-modified oligonucleotides	Direct effect in chimpanzee model in reducing HCV replication and viraemia
miR-196	Selective target for intervention	Implications for treatment
miR-26a	Deliverable to HCC sites using adeno-associated virus serotype 8	Decreased proliferation and induced tumour-specific apoptosis
miR-124	Induces tumour-specific apoptosis	Prevents and suppresses HCV development in murine model

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; miR/miRNA: MicroRNA; IL: Interleukin.

miR-122 and their expression is up regulated in primary human HCC due to decreased miR-122 levels[115,147]. Metastatic HCCs also show significantly lower let-7g levels, a miRNA that targets type I collagen a2 and when present at normal levels should prevent HCC spread[148].

The hepatocyte growth factor (HGF)/c-Met signalling cascade is considered a key pathway in HCC metastasis[134]. HGF interacts with the c-Met receptor tyrosine kinase to increase cell motility and invasion, while also conferring apoptotic protection. c-Met is associated with aggressive HCC and poor outcomes, and is regulated by miR-1, -34a, -23b and -199-3p levels of which are low in HCC[134]. Silencing of miR-1 inhibits HCC cell growth, and increases cell invasion, through c-Met down regulation[127]. Ectopic expression of miR-34a prevents HCC invasion and migration by reducing c-Met-induced phosphorylation of extracellular signal-related kinases 1 and 2 in HepG2 cells[149]. Likewise, over expression of miR-23b reduces levels of c-Met and urokinase-type plasminogen activator, a downstream target of HGF/c-Met signalling; this inhibits HCC proliferation and migration[150]. Regulation of cell cycle progression by restoring miR-199-3p levels to normal leads to induction of G1-phase cell cycle arrest (miR-199-3p targets c-Met and mTOR) thereby decreasing HCC cells' invasive ability[121]. Finally, miR-101 is also downregulated in HCC and reduces HGF-induced cell invasion and migration *via* inhibition of FOS oncogene expression[151]. Taken together these studies highlight how miRNAs control the central processes of invasion, metastasis and apoptosis that contribute to malignant transformation and tumour progression.

miRNAs AS DIAGNOSTICS FOR HCC

miRNAs are predominantly down regulated in tumour tissues[152], a pattern also seen in HCC. Several issues affect the identification and quantification of aberrantly expressed miRNAs in clinical samples confounding their potential as biomarkers. Despite these issues, several consistently dysregulated miRNAs have been identified in HCC (Table 2). Numerous studies have shown that circulating miRNA levels are altered in HCC progression. For example, serum miR-221 concentrations are 4.8-fold higher in HCC patients; high miR-221 levels correlate positively with cirrhosis, tumour size and tumour stage, and negatively correlate with overall survival[153]. Currently, there are few clinically useful serum HCC markers; α-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3) and des-γ-carboxyprothrombin (DCP) are of limited use[154]. The American Association for the Study of Liver Diseases discarded AFP as a marker for HCC surveillance and diagnosis in its July 2010 Practice Guidelines, highlighting the need for new biomarkers. miRNAs may have this potential. However, their use is complicated by the need for appropriate controls, as HCC usually develops from an underlying liver condition. For example, one study compared the miRNA expression profiles of three patient groups: one with HCC, one with chronic liver disease and one consisting of normal controls[155]. This study also showed that serum miR-16 and miR-199a concentrations were reduced and significantly associated with HCC[155]; of potential clinical relevance, miR-16 was more sensitive for detection of HCC than the three currently used biomarkers. Overall, these findings show the feasibility of miRNAs as serum markers for diagnosis of HCC. Should they continue to outperform current HCC markers in further studies, circulating miRNAs could be used in first-line testing of HCC patients. However, the study of circulating miRNAs as HCC biomarkers is a relatively recent concept, with further studies and validation of results in larger patient cohorts needed before miRNAs are used in the clinical setting. In particular, the discovery of a miRNA which sensitively and reliably diagnose early stage HCC would greatly enhance their potential for clinical use.

miRNA expression profiles can also be used to assess prognosis. For example, low miR-26 expression is associated with high interleukin-6 expression and shorter survival[156]; better response to interferon treatment also occurs in patients with low miR-26 levels. Furthermore, a 20-miRNA signature which accurately predicts survival and recurrence of HCC has been developed[157]. These studies suggest that miRNA profiling may play an impor-

tant role in HCC management in the clinic, both for classification of HCC into subtypes determining treatment and in assessment of prognosis. Patterns of dysregulated miRNAs distinguish tumours based on molecular characteristics. For example, β-catenin mutation is associated with reduced miR-375 levels, and reduced miR-107 levels with HNF1α[158]. Such findings led to the proposal of a miRNA-based HCC classification system[159]; this could be used to determine treatment allocation, based on molecular pathology.

miRNAs AS THERAPEUTICS FOR HCC

Efficacy of miRNA-based gene therapy in HCC treatment has been demonstrated (Table 2). In one study, miR-122 expression was inhibited in chimpanzees using SPC3649 LNA-modified oligonucleotides. As miR-122 up regulates HCV replication in infected hepatocytes, its inhibition reduced HCV RNA production and decreased viraemia[160]. A phase I trial for SPC3649 (Miravirsen) resulted, becoming the first miRNA-targeted drug to enter human clinical trials. Miravirsen was well-tolerated and is currently undergoing phase Ⅱ trials in HCV null responders to pegylated interferon-α and ribavirin. However, issues regarding possible viral escape are arising, with one study showing that mutations in the miR-122 binding site in HCV 5'-UTR decreases Miravirsen efficacy[161]. Similarly, therapeutic miR-196 targeting has been investigated, with the results of these and similar studies likely to have significant implications for future treatment of HCV infection and HCC[162]. Recently it was demonstrated that HNF4α, a key regulator of hepatocellular carcinogenesis, becomes stably inhibited during hepatocellular transformation. Perturbation of this event through miR-124 systemic administration can prevent and suppress HCC development in a murine liver cancer model by inducing tumour-specific apoptosis without toxic side effects[163]. Thus miR-124 has therapeutic potential for treating liver cancer.

Several virally-delivered "classical" gene therapy products developed for HCC are currently progressing through clinical trial phases; however, virus-delivered miRNA-based gene therapies have yet to be tested in clinical trials[2]. Accurate assessment of this method's potential risks must be performed before further progress can be achieved. Nevertheless, early results from studies investigating the therapeutic delivery of miRNAs are showing promise. One such study in mice used self-complementary AAV serotype 8 (scAAV8) to deliver miR-26a to the HCC site; this delivery restored miR-26a expression in HCC cells, specifically decreasing cancer cell proliferation, inducing tumour-specific apoptosis, and protecting from HCC progression without toxicity[112]. 80% of treated mice had no or small tumours at 3 wk post-transduction, while most liver tissue in the untreated control group was replaced with HCC tumours. This study is of critical importance to the future of HCC treatment in that it was the first to demonstrate the therapeutic potential of restoration of expression of a dysregulated miRNA in the liver. Despite this, the relevance of therapeutic miRNA delivery to human HCC patients remains to be determined, emphasising the considerable amount of research needed in this field before clinical applications can be made. Nevertheless, the early successes of RNA-based therapies in clinical trials demonstrate that miRNAs and their inhibitors show great therapeutic promise for HCC. Future studies will no doubt shed light on how best miRNAs have the potential to alter survival rates of HCC patients.

Findings have also pointed towards long non-coding RNAs (lncRNA) as important tumorigenic candidates actively involved in gene regulation, with lncRNAs suggested as a link in carcinogenesis. Morover, lncRNAs can act as negative regulators of miRNAs and therefore may become important factors to consider when developing miRNA therapeutics. Several reports demonstrate an association of lncRNA with the development, progression, metastasis and poor prognosis in HCC patients[164-168].

CONCLUSION

In summary, studies have demonstrated unequivocally that miRNAs are important modulators of mRNA and protein expression. They are known to be involved in a variety of biological and pathological processes, such as the regulation of iron homeostasis and in HCC development and progression. As predicted by bioinformatic analysis and confirmed by numerous studies, some miRNAs target multiple genes involved in HCC progression. Similarly, several miRNAs often regulate a single aberrantly expressed gene. From these findings, we see that HCC progression is determined by a complex interaction of dysregulated miRNAs and their target mRNAs. This must be kept in mind when investigating the therapeutic potential of miRNAs, as changing the expression of a single miRNA may not be adequate to alter expression of the target gene.

Investigations into the potential clinical uses of miRNAs are ongoing, most notably in the early diagnosis and treatment of HCC. In addition, using miRNAs to subdivide HCC cases based on molecular pathology has been proposed; this system could also determine treatment allocation and aid in prognostic assessment. Overall, it seems likely that miRNAs will play an increasingly important role in the diagnosis and treatment of liver diseases associated with HCC over the coming years, leading to improved patient survival rates and better patient outcomes.

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P- Reviewers Cui GL, Ma L, Maruyama H, Tiribelli C
S- Editor Wen LL **L- Editor** A **E- Editor** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5227

World J Gastroenterol 2013 August 28; 19(32): 5227-5237
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Risk factors for local recurrence following neoadjuvant chemoradiotherapy for rectal cancers

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Received: April 5, 2013 Revised: July 3, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

Local recurrence (LR) has an adverse impact on rectal cancer treatment. Neoadjuvant chemoradiotherapy (nCRT) is increasingly administered to patients with progressive cancers to improve the prognosis. However, LR still remains a problem and its pattern can alter. Correspondingly, new risk factors have emerged in the context of nCRT in addition to the traditional risk factors in patients receiving non-neoadjuvant therapies. These risk factors are decisive when reviewing treatment options. This review aims to elucidate the distinctive risk factors related to LR of rectal cancers in patients receiving nCRT and to clarify their clinical significance. A search was conducted on PubMed to identify original studies investigating patients with rectal cancer receiving nCRT. Outcomes of interest, especially potential risk factors for LR in patients with nCRT, were then analyzed. The clinical importance of these risk factors is discussed. Remnant cancer cells, lymph-nodes and tumor response were found to be major risk factors. Remnant cancer cells decide the status of resection margins. Local excision following nCRT is promising in ypT0-1N0M0 cases. Dissection of lateral lymph nodes should be considered in advanced low-lying cancers. Although better tumor response resulted in a relatively lower recurrence rate, the evidence available is insufficient to justify a non-operative approach in clinical complete responders to nCRT. LR cannot be totally avoided by current multidisciplinary approaches. The related risk factors resulting from nCRT should be considered when making decisions regarding treatment selection.

Key words: Local recurrence; Rectal cancer; Neoadjuvant chemoradiotherapy

Core tip: This review identifies the distinctive risk factors associated with local recurrence (LR) in patients with rectal cancer receiving neoadjuvant therapy. These factors are different from the traditional risk factors seen in patients treated with surgery and/or adjuvant therapy alone. The clinical significance of these risk factors is clarified in detail. To our knowledge, no reviews concerning this topic have been published. The present manuscript might help to understand the origin of LR following neoadjuvant chemoradiotherapy and may receive attention from investigators devoted to improving the prognosis of rectal cancer.

Peng JY, Li ZN, Wang Y. Risk factors for local recurrence following neoadjuvant chemoradiotherapy for rectal cancers. *World J Gastroenterol* 2013; 19(32): 5227-5237 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5227.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5227

INTRODUCTION

Local recurrence (LR) is a major problem and threatens the prognosis of rectal cancer patients. For locally pro-

gressive tumors, LR can not be prevented just by improving surgical techniques. Therefore, preoperative, also known as neoadjuvant, therapy has been advocated due to its ability to down-stage tumors and thus increase resectability. Multidisciplinary neoadjuvant approaches have been proven to effectively control LR[1,2] and improve overall survival[3,4]. However, LR still occurs[5,6] and its pattern can change[7,8] with regard to time and location. For example, the time from operation to LR is prolonged[9]. Most importantly, neoadjuvant therapy and its downsizing effects on tumors have resulted in the emergence of some LR-associated risk factors unlike those related with only surgery plus adjuvant chemoradiotherapy, such as vascular invasion or tumor differentiation[8,10]. These distinctive risk factors, consisting of isolated remnant cancer cells and tumor response to neoadjuvant chemoradiotherapy (nCRT), have been reported to be associated with the prognosis of patients[11]. Therefore, determination of the characteristics of these factors and their clinical significance would provide very helpful data for clinical practice.

The aim of the present review was to characterize the risk factors in patients receiving neoadjuvant therapy, mainly nCRT. Moreover, the clinical implications of these risk factors in treatment decision-making following nCRT were also explored.

SEARCHING STRATEGIES AND SELECTING CRITERIA

A systematic review was performed in order to explore potential risk factors for LR following nCRT. A literature search was performed in PubMed and EMBASE databases for English-language papers published over the last 10 years, with outcome data limited to humans. The search terms used included "rectal cancer" or "rectal neoplasm"; "neoadjuvant" or "preoperative"; "radiotherapy" or "chemotherapy" or "chemoradiotherapy"; "recurrence" or "local recurrence" or "local control" or "local relapse" or "local failure" or "prognosis".

The criteria for including potential studies in the systematic review were: (1) randomized clinical trials (RCTs) or cohort studies investigating patients with rectal cancer receiving nCRT; (2) retrospective studies of LR in patients with rectal cancer who were treated with nCRT; and (3) studies evaluating parameters (risk factors) that may influence the outcome in terms of LR in patients with rectal cancer who were treated with nCRT. Articles that did not show LR or investigate the causes of LR were excluded. Furthermore, abstract-only publications and chapters from books were excluded. When the same series of patients were reported by the same authors in different articles, only the series with the longest follow-up was included in the review.

Two reviewers independently reviewed each article, and discrepancies were resolved by discussion and consensus. All data were extracted from the main text, tables, and figures of the articles. Traditional risk factors such as differentiation, vascular invasion, TNM staging and circumferential resection margin status were excluded. Risk factors related to the downsizing effect of nCRT were included.

Analysis of the data from the included studies was carried out. Descriptive statistics (simple counts, means, and medians) were either directly derived from the article or calculated based on the data presented in the article, and used to report studies, patients, and treatment-level data. Outcomes of interest, especially potential risk factors for LR in patients who received nCRT were synthesized by pooling relevant data, and then analyzed. Due to high heterogeneity among the studies and lack of RCTs, a meta-analysis was not deemed appropriate.

PATTERNS OF LR FOLLOWING nCRT

Time and location of LR

To better understand the risk factors, a deep insight into the patterns of LR is required. The patterns of LR can be described by two aspects, namely timing and location. The first aspect is the time interval to development of LR. Habr-Gama *et al*[9] found that the mean recurrence interval was 52 mo (18-79 mo) in 6 cases with sustained complete clinical response to nCRT. However, Coco *et al*[6] reported that the time to development of LR was longer than 5 years in approximately one third of cases treated with nCRT (4 of 14 cases). Similar results were observed in studies[12,13], in which only neoadjuvant radiotherapy (nRT) was administered. However, in a study which included patients receiving surgery alone or associated with postoperative chemoradiotherapy (pCRT) with an average follow-up of 10 years, LR occurred in 72% of patients within 18 mo of surgery[14]. These data suggest that neoadjuvant therapy may have an ongoing impact, different from that of pCRT, on the natural history of rectal cancer. This may be the reason why a better response can be induced by nCRT over time[15,16].

The second pattern is the subtle alteration concerning subsites of LR. It has been shown that the incidence of anastomotic recurrence is declining[12,17]. The two most common sites of LR in nCRT cases are the lower pelvis (56%) and presacral region (22%)[18,19]. Syk *et al*[20] indicated that the majority of LRs in patients receiving nRT were located anatomically below the S1-S2 interspace. The higher frequency of LR within the presacral area in patients undergoing nRT may be explained by the unique anatomical locations of the mesorectum and lateral lymph nodes (LLNs). The mesorectum is defined as the fatty and fibrous tissues surrounding the rectum. Most mesorectal tissues are located at the dorsal side of the rectum and include lymphatic and vascular vessels to which cancer may disseminate. Furthermore, a recent anatomical study revealed the presence of an alternative lymphatic drainage pathway from mesorectal LNs to LLNs[21] using three-dimensional reconstruction and histological section. This connection may provide a pathway for the cancer cells to spread or escape and LLNs may

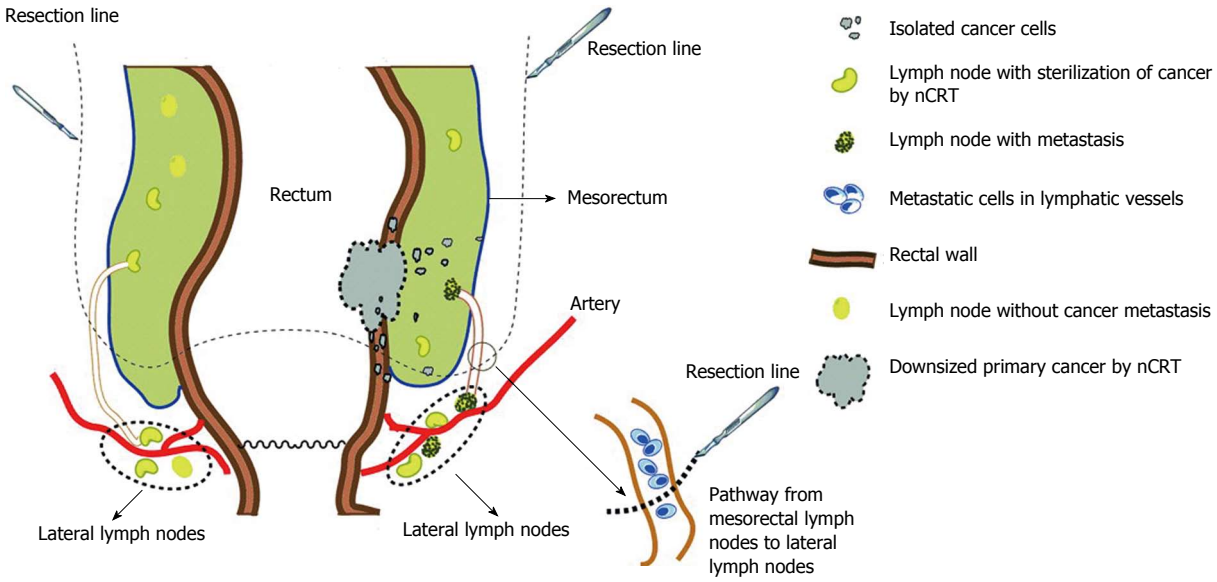

Figure 1 A diagram of risk factors for local recurrence in cases treated with neoadjuvant chemoradiotherapy. Resection line marks the resection range of a standard total mesorectum resection. nCRT: Neoadjuvant chemoradiotherapy.

serve as a harbor for these cells[22,23]. Some isolated cancer cells in the mesorectum or lymphatic tissues (see "Isolated tumor cells") serve as seeds for LR following nCRT. These cells are inhibited, but not killed, by nRT and rest in the G0 phase[24]. During surgery, cells may be spilled and implanted in the lower pelvis and presacral region resulting in LR.

We hypothesize that the seeds of LR may be the cancer cells at the margin of the mesorectum or within the lymphatic pathway from the mesorectum to LLNs. During a standard total mesorectum resection (TME), these cells may "leak" following complete resection of the mesorectum, implant in the presacral space due to the force of gravity and trigger subsequent LR (Figure 1). This hypothesis may be further confirmed if the tumor cells can be separated from post-operative lymph fluid drainage.

Clinical importance of follow-up

Understanding the altered LR patterns in patients with different neoadjuvant and intraoperative therapies has practical implications. On the one hand, delayed LR occurs in patients receiving nCRT, and thus, the standard 5-year follow-up currently recommended by the European Society for Medical Oncology[25] should be extended to at least 7-8 years and intensified monitoring is required in selected cases[26]. In addition, if delayed LR is expected to occur in a proportion of patients, the observational period in prospective and randomized trials[4,27] should be prolonged in order to draw more definitive conclusions. On the other hand, attention should be paid to common regions involved in LR in patients receiving neoadjuvant therapies which may help us accurately select the area at high risk for radiotherapy and avoid unnecessary irradiation.

ISOLATED REMNANT CANCER CELLS

As mentioned above, nCRT may be "suppressive" rather than "destructive" for a certain proportion of cancer cells. Thus, the surviving cells, if not removed by surgery, may restore their viability and evolve into seed cells for LR (Figure 1). These seed cells can be divided into two groups, extranodal and intranodal seed cells, according to their relationship with lymph nodes (LNs). Furthermore, two major types of LR derived from extranodal seed cells, tumor budding (inside the bowel wall) and mesorectal microfoci (MMF), have been reported, according to their locations.

TUMOR BUDDING

Relationship with LR

Tumor budding is described as a subset of isolated cancer cells located at the invasive front and extending from the neoplastic gland structures to the adjacent stroma[28]. Tumor budding has been reported to be an independent factor predicting prognosis[29,30]. Research on nCRT cases has shown that tumor budding is always described as isolated or small clusters of remnant cancer cells resulting from tumor regression. A control-case study[24] showed that nRT increased the frequency of budding cells compared with surgery without nRT (mean 54 *vs* 38, $P = 0.03$). These cells are always surrounded by fibrosis or an inflammatory reaction induced by nCRT. NCRT-induced tumor budding can be classified into two grades: high grade (clusters of budding cells easily observed by pathological examination) and low grade (minimal or isolated budding barely detected by pathological examination). According to Gavioli *et al*[31] study of 139 patients with nCRT, LR did not appear in the low grade budding group, while 8.8% of the high grade budding patients developed LR. In a more recent study, patients with low grade budding also had better 5-year disease-free survival than those with high grade budding (87.5% *vs* 55.6%, $P < 0.0001$).

Table 1 Intramural spreading distance after neoadjuvant therapy

Ref.	No. of patients	Neoadjuvant therapy regimen		Intramural spreading distance		
		Radiotherapy (Gy)	Chemotherapy	0-5 mm	6-10 mm	> 10 mm
Chmielik *et al*[32]	106	5 × 5	None	93	9	4
Chmielik *et al*[32]	86	50.4	5-Fu + LV	78	8	0
Mezhir *et al*[37]	20	50.4	5-Fu + LV	12	7	1
Guillem *et al*[36]	109	50.4	5-Fu + LV	108	1	0

5-Fu: 5-fluorouracil; LV: Leucovorin.

Clinical significance: decide the status of distal resection margin

It has been shown that the distal intramural spread of tumor budding is discontinuous in 57% of patients receiving nCRT[32]. The nature of this discontinuity is of special clinical importance; the supposed "clean" distal resection margin (DRM) in sphincter-sparing resection may not necessarily be free of cancer cells and longer a DRM may be required in a proportion of patients due to the possible existence of tumor budding. Thus, the focus is now "How far does tumor budding go?" Two studies demonstrated that DRMs less than 10 mm did not compromise LR[33,34]. In contrast, a study with a longer follow-up (5.6 years) demonstrated that a DRM less than 8 mm was associated with increased LR[35]. Why was there discrepancy between these two studies? First, the average period of follow-up may have had an influence. The follow-up time in these two studies may have been too short to draw definite conclusions (both were less than 36 mo). Second, the whole-mount section of the pathological examination was not used in these two studies, making the conclusion less convincing. Studies using whole-mount sections have shown that approximately 90% of patients receiving nCRT have a distal intramural extension of tumor budding within 5 mm, and 8% within 6-10 mm and less than 2% over 10 mm[32,36,37] (Table 1). Correspondingly, it has been suggested that the required length of the DRM should be shortened from 20 to 10 mm due to tumor remission induced by nCRT[36]. A DRM less than 10 mm is not yet justified for cases receiving nCRT based on current evidence. Therefore, following nCRT, the existence of budding cells is discontinuous and a supposed "negative" DRM less than 10 mm may not be a real negative margin for low-lying cancers.

MMF

Relationship with LR

Unlike tumor budding which is intramural, MMF, another risk factor for LR, is mesorectal. MMF is primarily defined as extranodal cancer deposits discontinuous with the primary tumor[38] in the mesorectum. The incidence of MMF is reported to be directly associated with the infiltrating depth of the primary tumor[38].

Ratto *et al*[39] specifically classified MMF into four major subtypes: endovascular (cancer deposits in blood vessels), endolymphatic (cancer deposits in lymphatic vessels but not in lymph nodes), perineural (cancer cell aggregates between the fasciculus and perineurium) and isolated (cancer deposits within the mesorectum, not a continuous extension from the main tumor mass). Clinically, MMF can be identified by careful pathological examination. Studies[39-41] have shown that MMF are detected in 13.8%-44.2% of cases after surgery despite downstaging induced by nCRT. Prabhudesai *et al*[38] reported that LR occurred in 17.2% (5/29) of patients with MMF and in 3.8% (1/26) of those without MMF, although the difference was not statistically significant.

Clinical significance: decide the status of circumferential resection margin and distal mesorectal margin

Similar to tumor budding, MMF may decide the status of the circumferential resection margin (CRM) and distal mesorectal margin (DMM) (Figure 2). However, no data are available regarding the appropriate CRM and DMM after nCRT. Should CRM and DMM be correspondingly shortened? Further pathological studies are required.

LYMPH NODES

Relationship with LR

Cancer cells harbored within LNs surrounding the rectum may serves as the seeds for LR. Although the nCRT-induced tumor regression does not necessarily parallel the sterilization of LNs metastasis, better tumor response may predict less LNs metastasis. Recent studies have proven that tumors at stage ypT0-1 correlate with a very low incidence of positive LN involvement[31,42-52] (Table 2). With regard to stage ypT2, LN involvement is present in about 20%-30% of cases[44,48].

Clinical significance: indication for local excision

With the belief that favorable tumor response may be equal to the disappearance of LNs metastasis, we propose that a proportion of pretreated T3 or T4 tumors might meet the requirements for local excision (LE). Several studies have shown that LR is not observed in ypT0 cases followed by LE, and the LR rate is around 3%-6% in ypT1 cases[53-60]. Moreover, the LR cases can be efficiently salvaged by subsequent radical dissection if early detection is achieved[54,61]. Therefore, LE is recommended by some authors for ypT0 or ypT1 cases due to its efficacy in local control which is equivalent to radical surgery[49,52,53,59,61-64]. Although these results are encouraging, the majority of the above-mentioned studies are retrospective and include small sample sizes. Thus, further

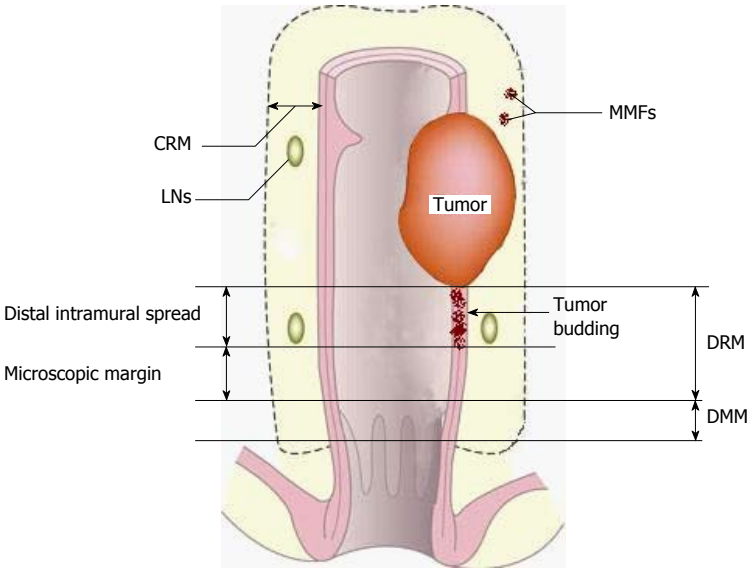

Figure 2 A diagram of resection margins of rectal cancer and their relationships with mesorectal microfoci and tumor budding. CRM: Circumferential resection margin; DRM: Distal resection margin. DMM: Distal mesorectal margin; LNs: Lymph nodes; MMF: Mesorectal microfoci.

Table 2 Association between ypT stage and ypN status *n* (%)

Ref.	No. of patients	Neoadjuvant therapy regimen		Time interval[1] (wk)	No. of patients with ypT0/T1	
		Radiotherapy (Gy)	Chemotherapy		ypN+/ypT0-1	ypN+/ypT2-4
Zmora *et al*[42]	109	45-50.4	5-Fu	6	4/33 (12.1)	30/61 (49.2)
Read *et al*[43]	644	20-45	5-Fu	NS	3/87 (3.4)	217/557 (39.0)
Bujko *et al*[44]	147	5 × 5		1	0/4 (0.0)	69/138 (50.0)
Bujko *et al*[44]	138	50.4	5-Fu	4-6	2/33 (6.1)	41/101 (40.6)
Pucciarelli *et al*[45]	235	45-50.4	5-Fu	6-8	3/69 (4.3)	45/166 (27.1)
Tulchinsky *et al*[46]	101	45	5-Fu	5-7	1/22 (4.5)	29/75 (38.7)
Habr-Gama *et al*[47]	401	50.4	5-Fu	8	3/25 (10.7)	75/224 (33.5)
Stipa *et al*[48]	187	50.4	5-Fu	NS	3/44 (6.8)	48/143 (33.6)
Kundel *et al*[49]	320	45	5-Fu	4-8	3/69 (4.3)	49/222 (22.1)
Gavioli *et al*[31]	139	50	5-Fu	4	2/34 (5.9)	38/105 (36.2)
Kim *et al*[50]	282	45	5-Fu	4-8	2/58 (3.4)	85/224 (37.9)
Lindebjerg *et al*[51]	135	60	5-Fu	8	8/47 (17.0)	32/88 (36.4)
Coco *et al*[52]	271	NS	NS	NS	3/71 (4.2)	70/200 (35.0)
Total	3109				37/596 (6.2)	828/2304 (35.9)

[1]Time interval refers to the time from the end of neoadjuvant therapy to subsequent operation. NS: Not specified; 5-Fu: 5-fluorouracil.

prospective, population-based and multi-center investigations are required to confirm these results.

With regard to ypT2 stage, 63% (53/88) of patients with ypT2 are reported to have at least one unfavorable pathological feature in addition to LNs metastases (vascular or perineural invasion, mucinous type and tumor size > 3 cm) for LE[65]. Perez *et al*[66] reported that the LR rate in patients with ypT2 who underwent LE was 9% (8/88) after nCRT. In cases with ypT3N0 or ypT4N0, the rate was up to 25% (14/25), including 14.7% (n = 8) systemic and 10.3% (n = 6) local relapse despite the absence of LNs micro-metastasis[66]. These findings indicate that ypT2-4 may have more residual cancer cells than detected and these tumor stages are not suitable for LE under the current nCRT regimen.

LATERAL LYMPH NODES

Relationship with LR

LLNs are a particular type of lymph nodes and dissection of LLNs is not included during regular TME. The incidence of LLN involvement varies from 7.7% to 20% in low and middle rectal cancer[67-69]. There is evidence to suggest that TME even with nCRT cannot completely remove remnant cancer cells in LLNs (Figure 1), especially in advanced tumors[45,70,71]. Kim *et al*[72] reported that 9 (7.9%) of 366 patients developed LR after nCRT and TME during a mean follow-up of 5 years, and lateral pelvic recurrence accounted for most (n = 24, 82.7%) of these cases. Patients with positive LLNs had a higher risk of lateral pelvic recurrence, compared with those with negative LLNs (LR rate: 26.6% *vs* 2.3%). Kusters *et al*[73] demonstrated that bilateral lateral lymph node dissection (LLND) generally resulted in better local control than unilateral LLND (LR rate: 15.4% *vs* 8.3%) in patients with advanced cancers after nCRT. When positive LLNs were detected preoperatively, the difference between unilateral and bilateral LLND was still significant (LR rate: 32.8% *vs* 14.2%). Furthermore, LR was detected on the contralateral side in a proportion of patients who

Table 3 Relationship between tumor response and local recurrence rate *n* (%)

Ref.	No. of patients	Neoadjuvant therapy regimen		No. of LR	
		Radiotherapy (Gy)	Chemotherapy	pCR LR/total	Non-pCR LR/total
Gavioli *et al*[31]	139	50	5-Fu	0/25 (0.0)	8/114 (7.0)
Stipa *et al*[57]	200	50	5-Fu	0/60 (0.0)	6/140 (4.3)
Hughes *et al*[71]	130	45	5-Fu	0/23 (0.0)	23/107 (17.7)
Kim *et al*[82]	114	50.4	5-Fu	0/10 (0.0)	17/104 (16.3)
Kuo *et al*[83]	248	50	5-Fu	2/36 (5.6)	66/212 (31.1)
Chan *et al*[84]	128	50	5-Fu	0/32 (0.0)	24/96 (18.4)
García-Aguilar *et al*[86]	168	40-65	5-Fu	0/21 (0.0)	7/147 (5)
Wheeler *et al*[87]	63	45-50	5-Fu	1/29 (3.4)	8/34 (23.5)
Theodoropoulos *et al*[88]	88	45	5-Fu	0/16 (0.0)	3/72 (4.2)
Total	1278			3/252 (1.2)	162/1026 (15.8)

LR: Local recurrence; pCR: Pathologic complete remission; 5-Fu: 5-fluorouracil.

underwent unilateral lymph node dissection. These data indicate that positive LLNs are a vital risk factor causing pelvic recurrence even after nCRT.

Clinical significance: application of LLND

There is controversy between Western and Japanese researchers concerning the application of LLND. Western researchers believe that nCRT plus TME may have a comparable outcome to that of LLND[74]. Moreover, resection of LLNs may result in injury to pelvic nerves. Thus, they recommend nCRT plus TME, not LLND. However, Japanese researchers indicate that LLND has a comparable outcome to that of nCRT plus standard TME regarding local control and the incidence of complications[75]. Thus, they recommend LLND. In our opinion, LLNs status is reflective of overall mesenteric LNs status and LLNs positivity may represent the poor response of rectal cancer to nCRT. LLND should be undertaken in selected patients, *e.g.*, those with tumor below the peritoneal reflection and poor tumor response. In addition, laparoscopic technology has unique advantages over laparotomy in terms of decreasing morbidity following LLND due to its high-definition close view in nerve-sparing.

TUMOR RESPONSES

Relationship with LR

A better tumor response may predict a more favorable prognosis for patients with advanced rectal cancer[76]. The response to neoadjuvant therapy includes remission in both primary tumor volume and lymphatic or vascular metastasis. Pathologic complete response (pCR) is defined as both ypT0 and ypN0, and the pCR rates range from around 10% to 30% in patients who underwent nCRT[77-80]. The final pathologic stage after nCRT and radical surgery is considered a vital factor in predicting LR. According to Mandard's Tumor Regression Grade (TRG) criteria[81], patients achieving a significant tumor remission (TRG1-3) displayed a relatively lower LR rate[71,82-87] compared with the non-downstaging group (TRG4-5). This figure decreased to 0%[31,71,82,86,88] (Table 3) in the pCR group. The reason for this may be that a pCR suggests a more favorable biological behavior and increases the chances of R0 resection. Moreover, complete regression of the primary cancer is paralleled with the disappearance of remnant cancer cells either in the mesorectum or lymph nodes[39].

Clinical significance: non-operative management

It has been shown that in patients with pCR, no residual cancer is found in resected specimens. This raises the question as to whether immediate radical surgery following nCRT is necessary, or, whether "watch and wait" is an appropriate strategy for these selected patients. Since pathological response can be judged only after tumor resection, a substitute parameter, clinical complete response (cCR), has been used to preoperatively screen potentially suitable patients[9,89]. A single-center study revealed that in patients treated with chemotherapy without surgery, only 5% of cCR cases (5 of 99) developed LR[9], whereas another study found that 8 of 10 patients had LR[90]. How do we explain such a big discrepancy? Actually, the critical premise for the "watch and wait" approach is to correctly identify the "real" suitable responders. A long-term persistent cCR may be a better representative of pCR. Only patients with sustained cCR for at least 12 mo were submitted to non-operative management in the study by Habr-Gama *et al*[9]. In contrast, the majority (75%) of patients with a short-term cCR (6-12 wk) were reported to have microscopic remnant cancers[70], at high risk of LR if subjected to "watch and wait". In addition, accuracy of staging in cases pretreated with nCRT is controversial. The absence of palpable tumors is not reliable evidence, nor is an invisible tumor on imaging methods, including transrectal ultrasonography, CT and MRI. Therefore, the overall attitude toward non-operative management remains critical and cautious, although the results from Habr-Gama *et al*[9,91] are promising. In our opinion, only selected cCR patients may undergo close observation without immediate radical surgery.

A CONTEMPORARY LOOK AT SURGERY-ASSOCIATED FACTORS

With the adoption of TME, LR and survival have im-

proved significantly in patients with rectal cancer, especially in those receiving anterior resection (AR)[92]. In comparison, abdominoperineal resection (APR) is reported to be related to a higher LR rate and poorer prognosis[93,94]. A possible explanation for the inferior outcome after APR is that surgeons often encounter more difficulties when resecting lower-lying tumors within a narrow pelvis[93]. Moreover, for those receiving nCRT, the appropriate surgical plane may be difficult to recognize due to tissue edema and fibrosis. These factors together may lead to inadequate excision of the mesorectum or of the tumor itself. In addition, the incidence of inadvertent intra-operative rectal perforation and post-operative anastomotic leak may increase, resulting in a higher LR rate[95-97].

With regard to AR, there is a legitimate concern about implanting exfoliated tumors cells when using circular staples. Despite the feasibility of low colorectal anastomosis, staples may also lead to implantation of viable tumor cells lying freely in the bowel lumen during staple firing[98,99]. That may also explain the mechanism of anastomotic recurrence in patients receiving nCRT (see Patterns of LR Following nCRT), who were expecting that tumor regression may translate to final sphincter-sparing surgery. Some authors[100,101] recommend intra-operative washout to eliminate exfoliated cancer cells because it is relatively risk-free and adds little to the operative trauma. However, it is difficult for surgeons to accomplish rectal washout in laparoscopic AR, as frequent laparoscopic manipulation probably increases tumor exfoliation, making wash-out even more crucial. Therefore, specific equipment or tools need to be designed to overcome the technical problems of laparoscopic rectal wash-out.

CONCLUSION

nCRT can downsize rectal cancer and facilitate subsequent radical resection. However, the impact of nCRT on downstaging of rectal cancer may also result in an altered pattern of LR and several distinctive risk factors for LR. These distinctive risk factors and altered patterns of LR are of clinical importance because they are decisive in treatment selection and follow-up. In future studies, we should not only identify but also improve our multidisciplinary approaches to minimize these factors.

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P- Reviewers Choi Y, Pottgen C, Sugimura H
S- Editor Wen LL **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5238

World J Gastroenterol 2013 August 28; 19(32): 5238-5249
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

DNA methylation in inflammatory bowel disease and beyond

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Author contributions: All the authors designed and wrote the manuscript.
Supported by National Institute of Health (DK80070, DK74454, and DK64289); the Eli and Edythe L. Broad Medical Foundation and American Gastroenterological Association Foundation to Mizoguchi E; and the Singapore A*STAR Graduate Academy (BM/AIF/13/001) to Low D
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Received: April 5, 2013 Revised: June 13, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

Inflammatory bowel disease (IBD) is a consequence of the complex, dysregulated interplay between genetic predisposition, environmental factors, and microbial composition in the intestine. Despite a great advancement in identifying host-susceptibility genes using genome-wide association studies (GWAS), the majority of IBD cases are still underrepresented. The immediate challenge in post-GWAS era is to identify other causative genetic factors of IBD. DNA methylation has received increasing attention for its mechanistical role in IBD pathogenesis. This stable, yet dynamic DNA modification, can directly affect gene expression that have important implications in IBD development. The alterations in DNA methylation associated with IBD are likely to outset as early as embryogenesis all the way until old-age. In this review, we will discuss the recent advancement in understanding how DNA methylation alterations can contribute to the development of IBD.

Key words: Intestinal inflammation; Crohn's disease; Colitis; DNA methyltransferase; Epi-therapy

Core tip: This review discuss the recent research advancement in the area of DNA methylation during the pathogenesis of inflammatory bowel disease (IBD) and IBD-associated cancer, with a focus on highlighting major players mediating DNA methylation alterations during IBD development. Temporal and spatial differential DNA methylation status that contributes to the disease, as well as epi-therapy treatment options for IBD patients, are also discussed. This emerging information will have important clinical significance, especially so in this post-genome-wide association studies era of IBD research.

Low D, Mizoguchi A, Mizoguchi E. DNA methylation in inflammatory bowel disease and beyond. *World J Gastroenterol* 2013; 19(32): 5238-5249 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5238.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5238

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory condition that affects the intestine of millions of individuals throughout their lifetime[1]. IBD is classified into two major forms, Crohn's disease (CD) and ulcerative colitis (UC), which both exhibit etiologically and clinically distinct features. Patients with IBD have a 2-3 fold greater life time risk of developing IBD-associated colorectal cancer (IBD-CRC)[2]. Although numerous clinical and experimental reports have given large amount of insights on the pathogenesis of IBD, the complexity of the initiation of IBD renders an incom-

plete understanding. Recently, there has been significant progress in identifying risk loci that are associated with IBD patients through genome-wide association studies (GWAS). These robust analyses have identified 163 IBD susceptible gene loci[3-6]. Genome-wide meta-analysis has confirmed that 71 of these loci are associated with CD, but only accounts for 25% of disease heritability[4]. The immediate challenge of the post-GWAS era is to unravel other parameters that may be less obvious from a genetic point of view. One of such emerging fields is epigenetics, in particularly DNA methylation. In this review, we will discuss the recent progress in DNA methylation analysis in IBD and how it can be used as a potential therapeutic target.

DNA METHYLATION ENSEMBLE IN IBD

By definition, epigenetics refers to a heritable change in gene expression phenotype that does not involve alterations in DNA sequence. DNA methylation, histone modifications and non-coding RNA are the three major components involved in epigenetic mechanism. In DNA methylation, the addition of a methyl group at the 5th position of cytosine (5mC) is common on CpG dinucleotides in eukaryotic genomes[7]. Methylation of CpG rich regions (CpG islands) are relative lower and are usually associated with transcription silencing when the methylated CpG islands occur at gene promoters[8]. DNA methylation is catalyzed by enzymes known as DNA methyltransferases and the reaction is reversible. Methyl groups can be edited and removed *via* actions of DNA demethylases during specific time points such as gametogenesis and disease onset including IBD. In this section, we discuss the roles of players mediating DNA methylation in the context of IBD development.

DNA methylation authors

Highly heritable and bona fide DNA methylation is attributed towards the actions of DNA methyltransferase. In the pathogenesis of IBD and IBD-CRC, three major DNA methyltransferases (DNMT) have been proposed to be involved, including DNMT1, DNMT3a and DNMT3b (Figure 1).

DNMT1 is a key maintenance methyltransferase that primarily methylates hemimethylated DNA in the genome during DNA replication. During IBD and IBD-CRC development, DNMT1 activity is significantly upregulated[9-11]. DNMT1 is highly expressed in actively inflamed colonic mucosa in UC patients as compared to normal or quiescent UC colonic mucosa[11]. In IBD-CRC, Foran *et al*[9] compared the methylation profiles of 36 IBD-CRC *vs* 44 sporadic CRC tumour specimens and demonstrated increased nuclear localization of DNMT1 in IBD-CRC than in sporadic-CRC, evidence linking inflammation-mediated DNMT1 activity. In addition, overexpression of DNMT1 is proposed to correlate with an abundance of CD68 positive macrophages, suggesting direct involvement of DNA methylation in a pro-inflammatory response[9]. Stimulation of HCT116 human colon cancer cells with interleukin (IL)-6 increases and stabilizes DNMT1 expression, leading to increase levels of global methylcytosine, especially at gene promoter regions[9]. This effect by IL-6 is mediated through AKT (Protein Kinase B), but not signal transducer and activator of transcription 3 (STAT3) or c-Jun N-terminal kinase (Jnk), pathway in Hela human cervical cancer cells[9]. Alternatively, another group showed that STAT3 binds directly onto the *DNMT1* promoter in malignant T cell lymphoma that is responsible for inducing DNMT1 expression[12]. All these suggest that specific cell type, temporal, or even inflammatory *vs* non-inflammatory mechanisms, affect DNMT1 expression and activity.

DNMT1 binds to non-intronic upstream enhancer of *Foxp3* (forkhead box P3), a locus required to induce the development of regulatory T cells (Treg) capable of suppressing broad ranges of inflammatory responses such as colitis[13]. Stimulation with IL-6 has been proposed to increase methylation in upstream enhancer regions of *Foxp3* in Treg cells, resulting in down-regulation of both mRNA and protein expression. This effect was not observed in STAT3-deficient Treg, providing additional evidence on the involvement of the STAT3-signalling pathway in the methylation process[13]. In a separate study, Li *et al*[14] reported that IL-6-associated STAT3 signalling is highly dependent on DNMT1 enzymatic activity. They showed that IL-6-induced DNMT1 expression results in hypermethylation on the promoter of suppressor of cytokine-signaling-3 (*SOCS3*), a negative regulator of IL-6 signalling. The decreased SOCS3 expression may then promote full pro-oncogenic effects of STAT3.

DNMT3 (includes three known members: DMNT3A, DMNT3B and DMNT3L) is another family of DNA methyltransferase. Although DNMT3 acts primarily for *de novo* methylation during gametogenesis and development, many reports have shown that DNMT3 can serve cooperatively with DNMT1 to regulate bona fide DNA methylation maintenance. Active UC colonic mucosa showed higher DNMT3B expression as compared to normal colonic samples or quiescent UC colon patient samples, but relatively lower than that of DNMT1[11]. Similarly, IBD-associated neoplasm lesions showed up-regulation of DNMT3B expression as compared to colonic epithelium without any neoplastic changes[15]. Conversely, human colorectal cancer cell lines (HCT15, DLD1, Col15, HT29, SW480 and RKO) are hypermethylated on the distal *DNMT3B* promoter as compared to healthy colon tissues, correlating it to the low expression level that results in hypomethylation of many of its target gene promoters[16]. These different observations may suggest that the etiology of IBD-CRC and sporadic-CRC are mechanistically distinct.

DNMT3A has been shown to play an important role in both innate and adaptive immune responses. For example, DNMT3A affects T cell polarization through *IL-4* and interferon gamma (*IFN*γ) promoter methylation upon ligation of T cell receptors[17]. In UC patient's

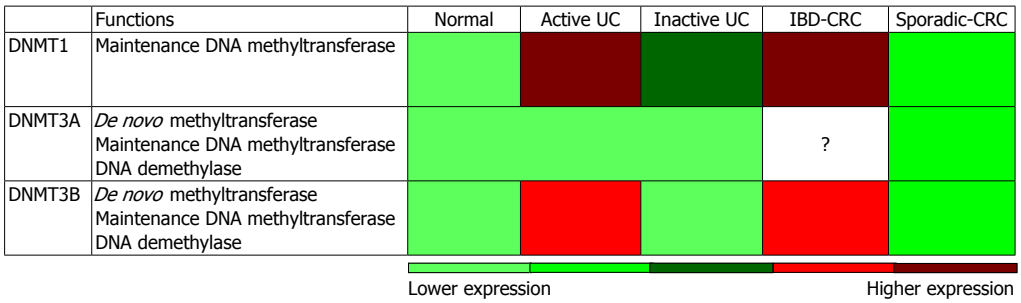

Figure 1 Potential relative expression levels of DNA methyltransferase in active-ulcerative colitis, inactive-ulcerative colitis, inflammatory bowel disease-associated colorectal cancer and sporadic-colorectal cancer patient specimens consolidate from several studies. DNA methyltransferase (DMNTs) is primarily responsible for DNA methylation maintenance, whereas DNMT3A/B have additional roles in *de novo* DNA methylation and demethylation functions. The relative DNMTs expressions were built on consolidated reports that were normalized to healthy controls to display potential relative expression in different inflammatory bowel disease associated diseases. UC: Ulcerative colitis; IBD-CRC: Inflammatory bowel disease-associated colorectal cancer.

peripheral T cells, levels of methylation within *IFNγ* promoter regions have been reported to correlate to the immune response against microbial antigens[18]. In addition, DNMT3A hypermethylates the CpG islands within the tumour necrosis factor alpha (*TNFα*) promoter region in the context of LPS stimulation[19]. However, another study has proposed no alteration of DNMT3A expression levels in colonic mucosa of UC patients[11]. It is possible that a modification of DNA methylation status during UC pathogenesis is mediated primarily *via* DNMT1 and DNMT3B. In contrast, meta-analysis of GWAS data has suggested *DNMT3A* as an important risk loci associated with CD[4]. Therefore, it is likely that methylation status in CD *vs* UC is controlled by different mechanisms.

In a clinical setting, the differential expression, involvement, and activities of DNMTs can provide additional options as a diagnosis marker tool to monitor IBD and IBD-CRC progression in patients.

DNA methylation editors

Editing and removal of methyl groups from 5mC can be actively or passively achieved through actions of DNA demethylases. Passive DNA demethylation blocks additional methylation during DNA replication by methylation dilution, or by inactivating DNMTs. Over the years, the search for active demethylases has been hindered by the fact that demethylation process is controlled by indirect multi-step mechanisms. DNA demethylation processes appear to be executed through DNA repair and base excision mechanisms, rather than direct removal of the methyl group from the 5mC moiety[20]. Recently, three proteins have been reported to potentially possess demethylase activity, including ten-eleven translocation (TET) methylcytosine dioxygenase, thymine DNA glycosylase (TDG), and activation-induced cytidine deaminase (AID).

TET converts 5mC to 5-hydroxymethylcytosine (5hmC) that is predicted to lift the repression of gene expression imposed by 5mC in both humans and mice[21]. Recently, Neves-Costa *et al*[22] demonstrated that TET1 negatively regulates the expression and secretion of a pro-inflammatory cytokine IL-1β in a THP-1 monocytic leukemia cell line. In addition, TET co-operates with TDG in the process of active DNA demethylation. TDG excises the mismatch bases at the deaminated 5mC or its derivatives caused by TET[23]. However to date, neither TET nor TDG has been implicated in the pathogenesis of IBD.

AID is another candidate involved in DNA demethylation[24,25]. AID belongs to the family of apolipoprotein B mRNA-editing catalytic polypeptide (APOBECs), which were extensively studied due to its master regulatory function in antibody diversification in B cells[26]. A process for this antibody diversification includes immunoglobulin class switch recombination (CSR), immunoglobulin somatic hypermutation (SHM), and gene conversion (GC)[27]. AID was originally demonstrated as an enzyme to convert cytosine (C) to uracil (U) for induction of SHM[28]. Subsequently, Morgan *et al*[24] unveiled an additional and unexpected ability of AID to convert 5mC to thymidine *in vitro* (5mC→T), suggesting the involvement of AID in DNA demethylation. This conversion of 5mC to T creates a T:G mismatch, which will be excised by T:G mismatch-specific glycosylases (*i.e.*, TDG). The T position will then be replaced with unmethylated C through base excision repair process, thereby concluding a 5mC to unmethylated C transition[29]. Recently, AID has been implicated in the pathogenesis of IBD and IBD-CRC[30,31]. Endo and colleagues showed that colonic AID expression is up-regulated under Th2-mediated colonic inflammatory conditions seen in T cell receptor (*TCR*)-α knockout mice[30]. In addition, ectopic expression of AID in colonic epithelial cells (CECs) was elicited in UC (54%) and IBD-CRC (80%) patients[30]. In contrast, AID expression was seen in only 40% of sporadic colon cancer, indicating the differential pathogenesis between IBD-CRC and sporadic-CRC with respect to AID functions. AID expression may be induced *via* IKK (IκB kinase)-dependent NF-κB signalling and further enhanced by Th2 cytokines such as IL-4 and IL-13[30]. Functionally, overexpression of AID in CECs has been reported to tremendously increase mutations within some, but not all, oncogenes including p53. Importantly, such mutations were significantly reduced in AID deficient mice[30,31].

However, it still remains largely unknown whether AID plays any specific roles in IBD and/or IBD-CRC through its epigenetic (demethylation) modification ability rather than its classical functions (SHM, CSR, and/or GC). The co-relationship between aberrant AID expression and IBD/IBD-CRC progression suggests that further studies on the role of AID-mediated epithelial homeostasis can potentially be translated into a therapeutic strategy for IBD patients by targeting AID.

In 2008, Kangaspeska *et al*[32] and Métivier *et al*[33] reported that DNMT3A and DNMT3B are recruited to gene promoters during transcription and they directly mediate cyclical demethylation and also remethylation processes. The identification of deaminase activity in DNMT3A and DNMT3B has received tremendous attention in the epigenetic field. Since it is clear now that DNMT3A and DNMT3B have dual functions for demethylation and methylation, the idea of dynamic methylation patterns during transcription will be further discussed in the following section.

DYNAMICS OF DNA METHYLATION FROM AN IBD PERSPECTIVE

Covalent modification of DNA through the addition of methyl moieties on CpG dinucleotides is highly stable and conserved. These epigenetic marks, however, do undergo dynamic changes at specific time points, including embryonic development and during perturbed cellular homeostasis such as increased cellular stress and disease onset. Thus, these temporal changes will have important implications that are relevant to the development of IBD.

During germ cell specification and post-fertilization, 5mC undergo *de novo* erasure and subsequent reprogramming[34]. The consequences of such wholescale DNA methylation reprogramming include formation of parental specific gene expression, including X-linked effects and genomic imprinting, of which gene expression are predominately contributed by specific parental allele. Several lines of evidence have demonstrated the parent-of-origin effects in IBD. As one of the earliest reports, Akolkar *et al*[35] demonstrated a familial association of IBD. In this study, clinical data analysis of 135 families showed that offspring of IBD affected mothers had higher risk for CD than offspring of fathers with IBD ($P = 0.00001$). Indeed, sex of parent seemed to play a role in IBD susceptibility and genetic imprinting process, at least in part, by DNA methylation. Fransen *et al*[36] recently present limited evidence for genomic imprinting effects of IBD susceptibility genes. They analysed 28 IBD susceptibility gene locus and found that *IL12B*, PR domain containing 1 (*PRDM1*) and nucleotide-binding oligomerization domain containing 2 (*NOD2*; L1007fs variant) have genomic imprinting effect. Recently, Schaible *et al*[37] showed that the offspring from female mice fed with methyl donor supplements (folic acid, betaine and vitamin B12) had a striking susceptibility towards dextran sulfate sodium (DSS)-induced colitis as compared to control mice fed with regular diet. These effects were also reflected with colonic mucosal DNA methylation profile alterations and prolonged gene expression changes, as well as difference in bacteria microflora when compared to mice with control diet. Therefore, better characterization of the effects and mechanisms of imprinting and parent-of origin can be utilized as a clinical risk predictor of IBD for offsprings of IBD susceptible parents in the future.

Another incidence where DNA methylation dynamics is activated is when colonic cellular homeostasis is perturbed such as during oxidative stress, which results in global loss or gain in DNA methylation. Oxidative stress and damage are common phenomenon in IBD and IBD-CRC that are mainly contributed by the reactive oxygen species produced by inflammatory cells[38,39]. Oxidative damage in cells induces recruitment of DNMT1 to the affected chromatin and forms a complex consisting of DNMT3B and members of the polycomb repressive complex 4, including Sirtuin-1 (SIRT1), Enhancer of zeste homolog 2 (EZH2) and embryonic ectoderm development (EED), to re-establish DNA methylation pattern after the DNA is repaired[40]. These key components re-localize from non-GC-rich regions to GC-rich regions[40]. The observation was validated in an *in vivo* model of colitis where infection with human commensal enterotoxigenic *Bacteroides fragilis* (ETBF) into a mouse model of adenomatous polyposis coli in Multiple intestinal neoplasia (Min) mice induced inflammation and tumorigenesis[40]. This model may provide a good putative explanation on the mechanism of how certain specific genes are hypermethylated, whereby other loci are hypomethylated, within the same cell, during disease onset.

Despite well-established consensus that DNA methylation is a highly stable modification on the DNA under steady state, two recent reports have changed this conventional perspective. In the first report, Kangaspeska *et al*[32] showed that estrogen receptor α (ERα) induces waves of transcription of its target promoter that involves series of active and cyclical demethylation and remethylation during the course of transcriptional activation. DNA methylation status were quantified using glutathione S-transferase tagged methyl binding domain (GST-MBD) pull-down assay, which showed a periodicity of 100 min at the ERα target *pS2* gene promoter. The second report by Métivier *et al*[33] showed similar cyclical demethylation-methylation effects at the *pS2* promoter, and further provided evidence of DNMTs are present at the promoter during transcription activation and is involved in both demethylation and remethylation processes. Specifically, methylated CpG were deaminated by DNMT3A and DNMT3B, resulting in a base-pair mismatch that is subsequently repaired by base-excision machinery. These two reports pioneered a previously unreported cyclical methylation-demethylation association with transcription and that this process is mediated by DNMTs, proteins previously tightly linked to only methylation but not demethylation. The authors have validated this observation in other promoters including *ER*α, trefoil

factor 3 (*TFF3*), and potassium inwardly-rectifying channel, subfamily J, member 8 (*KCNJ8*). Interestingly, these selected validated genes have previously been implicated in different studies of IBD and IBD-CRC in patients or animal models[41-44]. TFF3 is secreted by intestinal goblet cells that forms part of the enteric mucus layer and has a role in epithelial repair and restitution[45]. *TFF3*$^{-/-}$ mice are less reactive towards mounting repair response during colonic injury induced by chemical, hypoxia and radiation stress[46-48]. Another of the validated candidates is KCNJ8 (also known as KIR6.1), which forms the pore-forming sub-unit of the ATP-sensitive potassium channel (K_{ATP}). hydrogen sulphide (H_2S), produced by colonic smooth muscles, neurons and other enteric cell types, activates and opens K_{ATP} channels in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced murine colitis model[44]. Similarly in TNBS-induced colitis in rats, the production and effects of H_2S is associated with the resolution of colitis[49]. Nevertheless, whether cyclical demethylation-remethylation process plays any roles in the pathogenesis of IBD remains elusive, and further extensive studies will be required.

IMPACT OF ENTERIC MICROBES IN IBD HOST DNA METHYLATION

It has become increasingly apparent that dysregulated host microbial interactions contribute to the induction, exacerbation and perpetuation of IBD. Importantly, commensal microbes have an ability to alter DNA methylation status. Mice that were housed in germ free (GF) conditions exhibited hypermethylation of the chemokine ligand *CXCL16* [chemokine (C-X-C motif) ligand 16] in the colon, as compared to mice kept under specific pathogen-free (SPF) environment[50]. CXCL16 expressed on the surface of antigen-presenting cells, including subsets of CD19+ B cells and CD14+ monocytes/macrophages, mediates the adhesion and phagocytosis of gram-negative and positive bacteria[51,52]. Soluble CXCL16 can also act as a strong chemo-attractant for CXCR6+ [chemokine (C-X-C motif) receptor 6] T cells[53,54]. Up-regulation of CXCL16 mRNA and protein has been reported in CD patients[55]. Hypermethylation of *CXCL16* gene in GF mice leads to the gene activation and accumulation of invariant natural killer T (iNKT) cells, in the colonic lamina propria. iNKT cells are highly conserved subset of T cells expressing a semi-invariant T cell receptor, which is restricted to CD1d and specific for the glycosphingolipid antigen α-galactosylceramide. Furthermore, the activated CXCL16 pathway made GF mice more susceptible against oxazolone-induced Th2-type of acute colitis as compared to SPF mice[50]. Importantly, colonization of neonatal GF mice with a conventional microbiota reduced hypermethylation of *CXCL16* to SPF level[50]. However, this phenomenon was not observed when adult GF mice were colonized with the same conventional microbiota, indicating that early-life microbial exposure has a significant impact on host epigenetic status[50]. In addition, recent studies showed that oral inoculation of lipoteichoic acid (LTA)-deficient *Lactobacillus acidophilus* bacteria (NCK2025), protect mice from colitis-associated cancer presumably by restoring aberrant DNA methylation pattern of cancer-specific genes[56,57]. LTA is a major immunostimulatory component of cell wall of Gram-positive bacteria, which can specifically bind to CD14 and toll-like receptors (TLRs) such as TLR2 on host cells. It is well known that host-microbial recognition is attributed to TLRs. Of note, TLR2 deficient (*Tlr2*$^{-/-}$) mice were characterized by low abundance of intestinal *Firmicutes* and high proportion of *Proteobacteria*, *Bacteroidetes* and *Actinonbacteria*, as compared to wild-type mice[58]. This specific change in microbial composition was associated with epigenomic alterations. For instance, 1.4% of the interrogated genome in *Tlr2*$^{-/-}$ mice was differentially methylated[58]. Female wild-type C57BL/6J mice that were given methyl-donor supplemented diet produce offspring that exhibit different microbiome profile at postnatal day 30, as compared to control diet offspring[59]. All these data cumulatively suggest that the commensal microbiota can directly influence the status of host DNA methylation and therefore may have important implications in IBD development.

In addition to how bacteria affect the host DNA methylome, the status of DNA methylation on exogenous sources of DNA, in this case bacterial DNA and host self-DNA, also plays a role in the pathogenesis of autoimmune diseases such as IBD. Bacterial DNA has high CpG frequencies but is predominately unmethylated and has immunostimulatory effect[60]. It was originally shown that the introduction of bacterial CpG motifs oligodeoxynucleotides exacerbates existing intestinal inflammation in DSS-treated mice[61]. Recent studies showed that the unmethylation status of bacterial DNA is the predominate factor to induce human plasmacytoid dendritic cells to produce high levels of interferon-alpha (IFN-α), since methylation of the bacterial DNA abolished this induction[62]. These unmethylated CpG DNAs are recognised by the host toll-like receptor 9 (TLR9)[63]. Specific CpG motifs (purine-purine-CpG-pyrimidine-pyrimidine) common in microbial DNA, but which are rare in mammalian DNA, have the strongest activation potential of TLR9[64]. In contrast to bacterial DNA, mammalian DNA has lower CpG frequencies and is predominately methylated, with an exception of CpG islands. There are now increasing evidence that these mammalian self-DNA, presumably released from necrotic cells, can also be an effective TLR9 ligand[64]. Under normal circumstances, the host immune system is protected against self-DNA because of the intracellular location of TLR9. However, during IBD progression, natural antimicrobial peptide LL37 is expressed on the mucosa surfaces and form an immuno-complex with self-DNA, which may lead to the activation of TLR9[65]. Yasuda *et al*[64] showed that CpG-rich DNA from mammalian DNA, commonly found on CpG islands, are optimal sequence to activate TLR9 and suggested a possible contribution towards

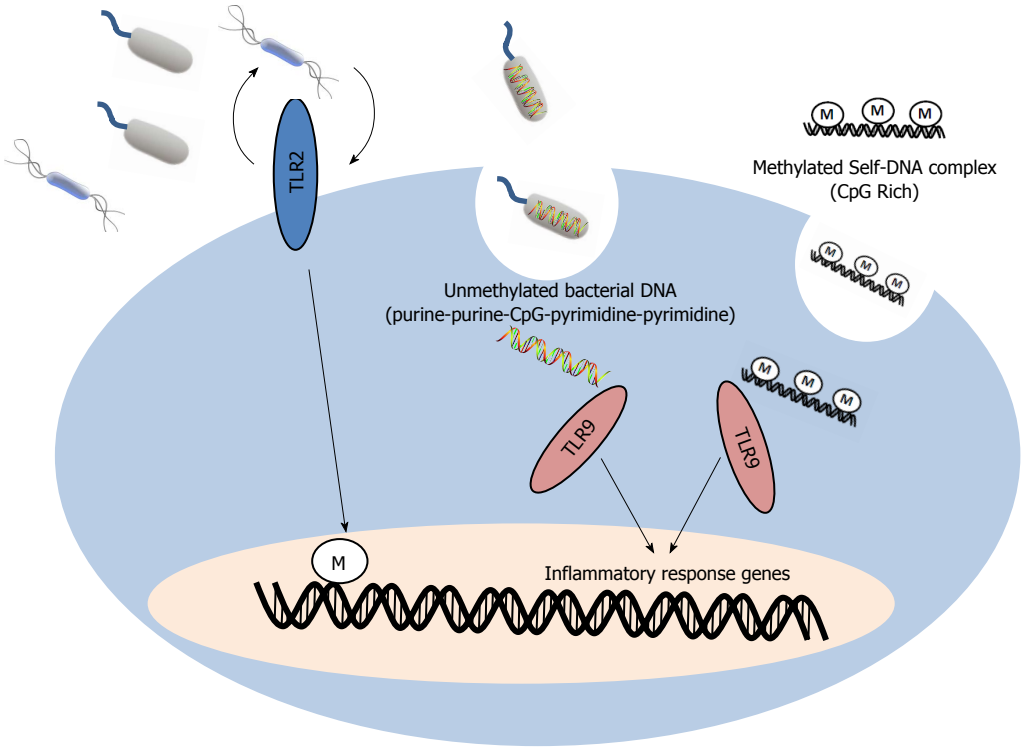

Figure 2 Host genetics and epigenetics alterations by commensal bacterial and self-DNA. Alterations in intestinal microflora or host pathogen recognition functions, such as toll-like receptor (TLR)2, directly affect host DNA methylation. Endocytosis of bacterial and release of unmethylated bacterial DNA into host cell triggers inflammatory response *via* TLR9. Strong activation requires a purine-purine-CpG-pyrimidine-pyrimidine bacterial DNA motif. Endocytosis of CpG rich methylated self-DNA also activates TLR9 to induce similar inflammatory response *via* TLR9, but with on a less magnitude compared to stimulation *via* bacterial DNA.

autoimmune diseases pathogenesis. However, this TLR9 activation by methylated self-DNA is still comparatively lower than those of unmethylated bacterial DNA[62]. As such, it was proposed that the initiation of autoimmune disease, such as IBD, is initiated by unmethylated microbial DNA whereas subsequent autoimmunity is mediated by methylated (or unmethylated) self-DNA[62]. Therefore, appropriately targeting self-DNA mediated immune responses may be another attractive option to reduce the perpetuation of inflammation in IBD.

In summary, bacterial genetics have a direct impact on host epigenetics. Similarly, bacterial and host (self-DNA) epigenetics can also directly affect host genetics to trigger inflammatory responses (Figure 2).

GENOME-WIDE DNA METHYLOME PROFILES IN IBD

Recent advances on genomic/epigenetic technologies targeting the "omics" level have contributed to a plethora of reports on genome-wide DNA methylome analysis to study the pathogenesis of IBD. The information derived from the analyses in IBD will provide significant rationale to open up a new avenue to develop novel diagnostic and therapeutic strategies. Indeed, genome-wide altered methylation patterns have been shown to be enriched around GWAS identified loci[66,67]. In addition, methylome profiling may also resolve the differences in etiology and pathogenesis of UC *vs* CD.

Nimmo *et al*[67] recently profiled the methylome of whole blood genomic DNA from 21 ileal CD patients and 19 healthy controls. They identified 1117 CpG sites that are differentially methylated. Within the list, 35 genes overlapped with previous GWAS identified CD loci, including *NOD2*, *TNF*α and caspase recruitment domain family, member 9 (*CARD9*). Comparative analysis of these gene hits showed that differentially methylated CpG sites are located within 25-100 kb of the 71 previously identified GWAS CD loci. Importantly, sex, environmental and individual lifestyle (*e.g.*, non-smoking and immunomodulatory therapy status) factors were taken into consideration for the selection of cohort in this study because these factors are influential in determining IBD, as well as epigenetic changes. This is especially apparent as seen from the high discordance rate of CD (68%) and UC (85%) in monozygotic twins, who had identical genomes[68]. The immediate question is how these identical genomes in monozygotic twins divert into different phenotypes outcome. A recent report studied 20 monozygotic twins discordant for UC and investigated the genomic profile based on three-layers of genome-wide scans, including transcriptome profiling, genome-wide methylation variable positions (MVPs) and genome-wide differentially methylation regions (DMRs)[69]. In this study, they identified 61 disease loci defined by differential gene expression profile and at least one MVP or DMR position within 50 kb from

Table 1 High throughput DNA methylome profiling in inflammatory bowel disease

Ref.	Disease	Tissue/cell	Array platform	Significant differential methylation	GWAS overlap
	Human patients				
Lin *et al*[73]	UC and CD	Intestinal	Illumina goldengate	7 CpG sites	Not reported
Cooke *et al*[66]	UC and CD	Rectal	Illumina infinium human methylation 27	3604 (UC) and 472 (CD) loci	Yes
Lin *et al*[72]	UC and CD	B cell	Illumina goldengate	24 (UC) and 14 (CD) CpG sites	Not reported
Häsler *et al*[69]	UC	Intestinal	Illumina human methylation 27 and nimblegen custom 385K	61 loci	No
Nimmo *et al*[67]	CD	Whole blood	Illumina human methylation 27	1117 CpG sites	Yes
	Mouse				
Kellermayer *et al*[59]	DSS colitis (postnatal day 30 *vs* day 90)	Colon	Custom array (Agilent)	271 intervals	Not reported
Kellermayer *et al*[58]	*Tlr2-/-*	Colon	Custom array (Agilent)	387 intervals	Not reported

UC: Ulcerative colitis; CD: Crohn's disease; GWAS: Genome-wide association studies; DSS: Dextran sulfate sodium.

the transcription start site. Promoter regions of these hits showed prominent hypomethylation, whereas gene-intronic regions were more frequently hypermethylated. However, none of these 61 loci overlapped with the previously reported 47 UC GWAS risk loci[5]. Nevertheless, environmental factors and lifestyle surely contribute to the pathogenesis of IBD and provide the most direct clues to understand how identical genomes from monozygotic twins can have distinct susceptibility to IBD. IBD usually occurs during young adulthood and the peak age of onset is around 15-30 years old[70,71]. Thus, identification of the changes in methylome during crucial developmental time point can provide great insights on IBD risk. Studies showed that postnatal day 90 mice had increase susceptibility to DSS-induced colitis as compared to postnatal day 30 mice[59]. Methylation specific amplification microarray (MSAM) revealed 271 differential methylation genomic intervals between the above two mice groups[59]. These results suggest that age-dependent methylation dynamics is another important aspect to consider in the risk of IBD.

In addition to prying into the individual genomic status in UC or CD as compared to normal individuals, epigenome-wide profiling can also dissect the differences in disease-associated loci between UC and CD. Cooke *et al*[66] recently characterized the genome-wide methylation changes in the rectal samples obtained from patients with inflamed UC/CD and non-inflamed UC/CD. Consistent with other reports, many identified loci in this study overlapped with GWAS-identified risk loci, including *CARD9*, intercellular adhesion molecule 3 (*ICAM3*) and cadherin 1 (*CDH1*). Inflamed UC and CD, as well as non-inflamed UC formed individual methylome signatures when compared to normal control individuals. Interestingly, there was no difference in the methylation profile between inflamed UC and inflamed CD. In contrast, 13 differentially methylated loci were identified between non-inflamed UC and non-inflamed CD. These multiple comparison suggests that the different sub-types of IBD, as well as disease severity, may be distinguished by their methylome status. In addition, Lin *et al*[72,73] also reported the methylome profiles of UC and CD patients derived B cells and intestinal tissues. Therefore, the methylome may be one of the useful clinical diagnostic biomarkers in IBD (Table 1). However, much more careful attention would be necessary in this regard because different cell types exhibit different methylomes in IBD.

EPI-THERAPY TARGETING DNA METHYLATION IN IBD

Several compounds targeting DNA methylation status has been demonstrated to have potential therapeutic effects on animal models of IBD and/or human IBD patients. One of these compounds is folate, a methyl donor that exerts an effect to increase global methylation. Chronic UC patients that were given dietary folinic acid, a vitamer of folic acid, supplementation (15 mg/d) had a lower risk of colon cancer[74]. Kominsky *et al*[75] recently also showed that intraperitoneal injection of folate (50 mg/kg) into DSS-treated mice results in less severe colitis. In addition, dietary folate deficiency led to aggravation of DSS-induced colitis in rats[76]. These results are consistent with clinical reports showing that folate deficiencies are common in IBD patients[77-79]. However, oral dietary supplementation of folate did not seem to have an effect on the suppression of IBD-CRC in an azoxymethane/DSS-associated cancer model[80]. In this model, diet supplementation with folic acid (8 mg/kg) did not show any alterations in intestinal microflora or difference in tumor initiation, growth and progression as compared to the control mice without receiving folic acid supplement. One possible reason for this failure is that the chronic inflammation that has transited into tumorigenic stage would have acquired more stable genetic changes including chromosomal instability and translocation, as well as genetic mutations, as compared to acute intestinal inflammation. These alterations in DNA sequence may occur at critical DNA methylated CpG sites and hence global methylation effects of folate can no longer re-establish methylation at these mutated CpG target sites.

Development of small compounds that can directly or indirectly affect DNA methylated mediated gene ex-

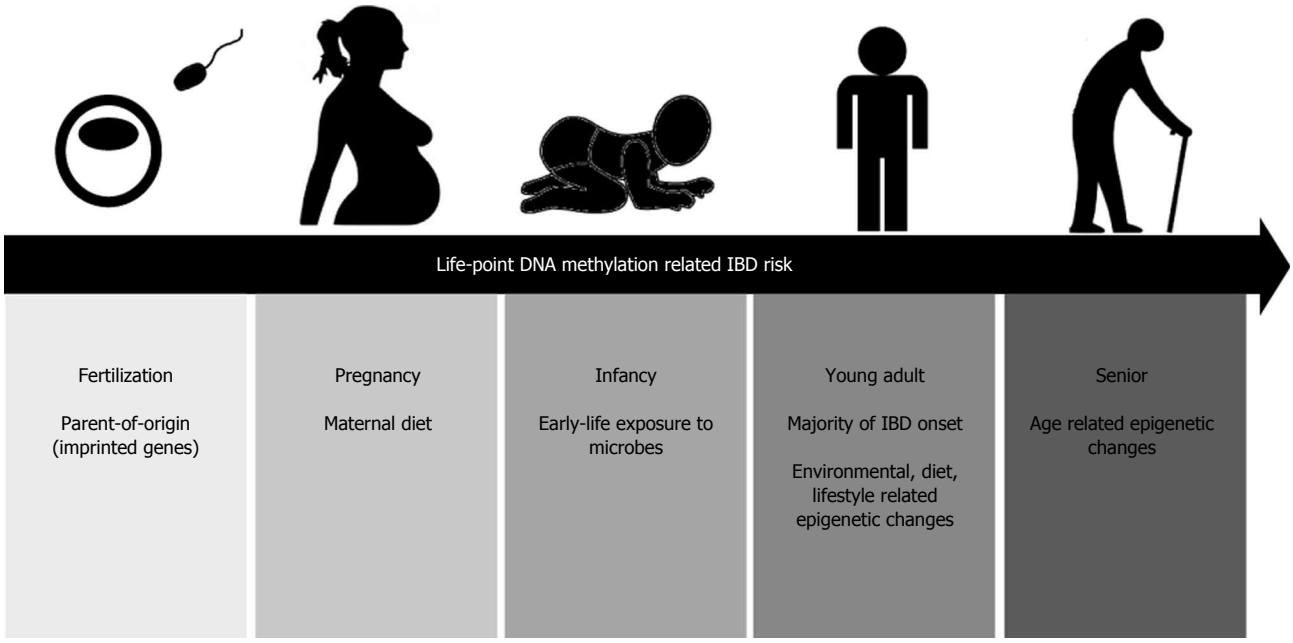

Figure 3 Life-stages with an impact on epigenetic changes that increase inflammatory bowel disease risk. Alterations in DNA methylome in inflammatory bowel disease (IBD) pathogenesis begin right from the fertilized egg. The risk alleles are inherited, and its expression is dependent on the parent-of-origin (imprinting). Maternal diet during pregnancy may also potentially alter the fetal IBD-associated-methylome. Exposures to certain microbes during infancy can also have lasting effects on DNA methylation alteration towards IBD susceptibility. Environment-, lifestyle- and diet- associated DNA methylation changes are important aspects during young adulthood where the majority of IBD onset occurs.

pression may also be useful targets of IBD treatment. Meng *et al*[81] demonstrate that using a combination of a novel tylophorine analog W-8, together with TGF-β (transforming growth factor), demethylates *Foxp3* promoter. Tylophorine analogs, including W-8, are phenanthroindolizidine alkaloids that have anti-cancer and anti-inflammatory effects. The effect of W-8 is mediated through ERK (extracellular signal-regulated kinase) pathway inhibition that results in the down-regulation of DNMT1 expression. Therefore, W-8 appears to up-regulate *Foxp3* expression by demethylating the promoter in the presence of TGF-β and promotes differentiation of naïve CD4+ T cells into Foxp3+ Treg cells with immunosuppression capabilities.

The challenge in developing innovative therapies for IBD has been on-going. Currently, oral and topical aminosalicylates are usually the first-line medication to treat IBD. Other immunosuppressive agents including azathioprine, methotrexate and cyclosporine are also in used. However, the beneficial effects of these drugs are accompanied with detrimental side effects, such as allergy. In addition, not all patients respond to these treatments. Recently, the use of anti-TNFα antibody has also been deployed to control IBD in patients. However, on top of the adverse side effects of anti-TNFα antibody, the administration of the treatment requires invasive intravenous infusion or subcutaneous injection and the high cost of this form of medication, which range from US$3000 to US$8000 per infusion, is a major disadvantage. Therefore epi-therapy drug design is an attractive alternative method to develop an effective, low-cost and non-invasive therapy for IBD patients.

CONCLUSION

DNA methylation has great heuristic potential in improving our understanding of the IBD pathogenesis in the post-GWAS era. Individuals who inherited a normal set of DNA may still be susceptible to IBD depending on epigenetic changes during their course of life. As described in this review, epigenetics changes that may account for IBD risk begin right from the fertilized egg to entire life period (Figure 3). Further advancements in this promising field would allow the discovery of new mediators to control DNA methylation/demethylation, aiming to improve the lives of patients with IBD and IBD-CRC.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Michael Choi for his helpful discussion and advice. We would like to thank Mr. Alan Kamba's assistance for preparing this manuscript.

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P- Reviewers Capasso R, Koyama N, Sipos F
S- Editor Zhai HH **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5250

World J Gastroenterol 2013 August 28; 19(32): 5250-5260
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice

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Supported by Research grants from University of Tsukuba, the Basic Research Support Program for Young Researcher
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Received: April 3, 2013 Revised: June 12, 2013
Accepted: July 4, 2013
Published online: August 28, 2013

Abstract

AIM: To investigate the role of human platelets in liver fibrosis.

METHODS: Severe combined immunodeficiency (SCID) mice were administered CCl_4 and either phosphate-buffered saline (PBS group) or human platelet transfusions (hPLT group). Concentrations of hepatocyte growth factor (HGF), matrix metallopeptidases (MMP)-9, and transforming growth factor-β (TGF-β) in the liver tissue were compared between the PBS and the hPLT groups by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The effects of a human platelet transfusion on liver fibrosis included the fibrotic area, hydroxyproline content, and α-smooth muscle actin (α-SMA) expression, which were evaluated by picrosirius red staining, ELISA, and immunohistochemical staining using an anti-mouse α-SMA antibody, respectively. Phosphorylations of mesenchymal-epithelial transition factor (Met) and SMAD3, downstream signals of HGF and TGF-β, were compared between the two groups by Western blotting and were quantified using densitometry. Hepatocyte apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling. Furthermore, the accumulation of human platelets in the liver 2 h after platelet transfusion was compared between normal and fibrotic livers by immunohistochemical staining using an anti-human CD41 antibody.

RESULTS: The fibrotic area and hydroxyproline content in the liver were both significantly lower in the hPLT group when compared to the PBS group (fibrotic area, 1.7% ± 0.6% *vs* 2.5% ± 0.6%, $P = 0.03$; hydroxyproline content, 121 ± 26 ng/g liver *vs* 156 ± 47 ng/g liver, $P = 0.04$). There was less α-smooth muscle actin staining in the hPLT group than in the PBS group (0.5% ± 0.1% *vs* 0.8% ± 0.3%, $P = 0.02$). Hepatic expression levels of mouse HGF and MMP-9 were significantly higher in the hPLT group than in the PBS group (HGF, 109 ± 13 ng/g liver *vs* 88 ± 22 ng/g liver, $P = 0.03$; MMP-9, 113% ± 7%/GAPDH *vs* 92% ± 11%/GAPDH, $P = 0.04$). In contrast, the concentration of mouse TGF-β in the liver tissue was significantly lower in the hPLT group than in the PBS group (22 ± 5 ng/g liver *vs* 39 ± 6 ng/g liver, $P = 0.02$). Phosphorylation of Met was more prevalent in the hPLT group than in the PBS group (37% ± 4%/GAPDH *vs* 20% ± 8%/GAPDH, $P = 0.03$). Phosphorylation of SMAD3 was weaker in the hPLT group than in the PBS group (60% ± 12%/GAPDH *vs* 84% ± 12%/GAPDH, $P = 0.1$), although this difference was not significant. Furthermore, a lower rate of hepatocyte apoptosis was observed in the hPLT group than in the PBS group (5.9% ± 1.7% *vs* 2.9% ± 2.1%, $P = 0.02$). Significant human platelet accumulation was observed in the fibrotic liver tissues, whereas few platelets accumulated in the normal liver.

CONCLUSION: Human platelets inhibit liver fibrosis in SCID mice. Increased concentration of HGF in the liver suppresses hepatic stellate cell activation, induces MMPs, and inhibits hepatocyte apoptosis.

Key words: Human platelet; Liver fibrosis; Hepatocyte apoptosis; Hepatocyte growth factor; Transforming growth factor-β; Matrix metallopeptidases

Core tip: We assessed the effects of human platelet transfusion on liver fibrosis. Severe combined immunodeficiency (SCID) mice were administered CCl_4 and either phosphate-buffered saline or human platelets. The effects of a human platelet transfusion on liver fibrosis and hepatocyte apoptosis were compared. The fibrotic area, hydroxyproline content, and α-smooth muscle actin expression were decreased in mice that received human platelet transfusions. Transfusion increased mouse hepatocyte growth factor (HGF) and matrix metallopeptidases (MMP)-9 levels in the liver and decreased mouse transforming growth factor-β. Furthermore, transfusion suppressed hepatocyte apoptosis. Human platelets inhibited liver fibrosis in SCID mice. Increased concentration of HGF in the liver suppresses hepatic stellate cell activation, induces MMPs, and inhibits hepatocyte apoptosis.

Takahashi K, Murata S, Fukunaga K, Ohkohchi N. Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice. *World J Gastroenterol* 2013; 19(32): 5250-5260 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5250.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5250

INTRODUCTION

Chronic liver disease and liver cirrhosis are major causes of morbidity and mortality worldwide. In chronic liver disease, normal repair of hepatocyte damage and tissue remodeling is lost, resulting in fibrosis and ultimately cirrhosis, which leads to portal hypertension, hepatocellular carcinoma, and lethal hepatic failure[1]. The most common etiological factors in chronic liver disease are chronic hepatitis C virus infection, excessive alcohol consumption, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis. Liver transplantation is the only curative approach, and specific treatments that stop progressive fibrosis are currently unavailable[1].

Liver fibrosis is characterized by the excessive production and deposition of the extracellular matrix (ECM) proteins, such as collagen, proteoglycans, fibronectins, and hyaluronic acids[2]. Accumulation of the ECM results in remodeling of the hepatic structure. Among the deposited ECM proteins, collagen type I is a major constituent, which is mainly produced by hepatic stellate cells (HSCs). Matrix metallopeptidases (MMPs) are the key enzymes responsible for the degradation of all protein components of the ECM[3]. Recently, it has been reported that hepatocyte apoptosis in cirrhotic liver induces HSC activation, which promotes liver fibrosis[4].

Liver cirrhosis has traditionally been viewed as an irreversible state in which the normal hepatocellular structures and organization are destroyed and fibrosis is firmly established. However, several reports have opposed this conventional concept. Lang *et al*[5] reported that blocking transforming growth factor-β (TGF-β) with small interference RNA suppressed HSC activation and decreased liver fibrosis in mice. Iimuro *et al*[6] showed that the delivery of MMP-1 attenuated established liver fibrosis in rats. In recent years, platelets have been shown to exert both anti-fibrotic and fibrolytic effects on the liver[7-10].

In this study, we transfused human platelets into severe combined immunodeficiency (SCID) mice to examine the effects of human platelet transfusion on liver fibrosis. This model was used for the following two reasons: first, there is no direct evidence that human platelets inhibit liver fibrosis. Second, because *in vivo* human studies are difficult, xenotransfusion of human platelets into SCID mice has been used to examine the functions of human platelets[11,12]. Using this model, we evaluated the effects of human platelet transfusion on liver fibrosis and hepatocyte apoptosis.

MATERIALS AND METHODS

Animals

Experiments were performed using 8-12-wk-old male C.B-17/lcr-scid/scid Jcl mice weighing 20-26 g (CLEA, Tokyo, Japan). Mice were maintained in a temperature-controlled room on a 12-h light-dark cycle with free access to water and standard chow. After an acclimation period of at least 7 d, mice were divided into two groups: CCl_4 plus phosphate-buffered saline (PBS) administration (PBS group), and CCl_4 plus human platelet transfusion (hPLT group). All experiments complied with the Guidelines for the Care and Use of Laboratory Animals (University of Tsukuba).

Models for liver cirrhosis

To induce liver fibrosis, each mouse received an intraperitoneal injection of CCl_4 (200 μL/kg body weight) in a 1:3 ratio with corn oil twice a week for 8 wk. PBS or concentrated human platelets was transfused once a week from weeks 5 to 8. A 500-μL aliquot of PBS or concentrated human platelets was injected into the retro-orbital vein one day after the administration of CCl_4. Mice were sacrificed 96 h after the final administration of PBS or human platelet transfusion, and livers were removed and divided into two samples; One liver section was fixed in 10% buffered formalin for subsequent immunohistochemical analysis, and the other section was snap-frozen in liquid nitrogen and kept at -80 ℃ until use.

Transfusion preparations

Human whole blood was obtained from healthy volunteers. Platelet-rich plasma was obtained by centrifuging anticoagulated blood containing acid-citrate-dextrose at a 1:4 volume ratio at 120 *g* for 10 min. Samples were then

centrifuged at 1000 *g* for 15 min, and resuspended in citrate buffer (120 mmol/L NaCl, 4.26 mmol/L $NaHPO_4$, 5.5 mmol/L glucose, 4.77 mmol/L sodium citrate, and 2.35 mmol/L citric acid at pH 6.5). Platelets were then suspended in PBS and counted using a hematology analyzer (MICROS abc LC-152; Horiba Ltd., Kyoto, Japan).

Transfusion conditions and flow cytometric analysis of transfused platelets

To determine the number of cells for transfusion, 2.5×10^8, 5.0×10^8, or 10.0×10^8 of human platelets were transfused into naive SCID mice, and the post-transfusion percentage of transfused platelets was measured after 6 h ($n = 3$). We examined at 6 h because a 10% increase in peripheral platelet count 6 h after platelet transfusion improved liver function of the patients with liver cirrhosis in our clinical study. Because it required approximately 15 mL of human whole blood to prepare 10.0×10^8 of human platelets, 10×10^8/body weight was determined to be the upper limit.

Peripheral blood was collected from the lateral tail vein. Blood samples were incubated for 30 min with a biotin-conjugated rat anti-mouse CD41 antibody (AbD Serotec, Oxford, United Kingdom) that specifically detected murine platelets. Samples were then washed in platelet HEPES buffer (137 mmol/L NaCl, 2 mmol/L KCl, 0.4 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose at pH 7.4) containing 10% acid-citrate-dextrose, and centrifuged at 500 *g* for 5 min. Supernatants were removed and the cells were resuspended in platelet HEPES buffer containing 10% acid-citrate-dextrose. Samples were incubated with a FITC-conjugated mouse anti-human CD41 antibody (Dako, Glostrup, Denmark) that specifically detected human platelets and streptavidin-phycoerythrin (PE)/Cy5 (Biolegend, San Diego, CA, United States) for 30 min and then analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, United States). The post-transfusion percentage of human platelets was defined as human platelets/(human platelets + murine platelets).

After 6 h, the post-transfusion percentages of human platelets in naive mice that received 2.5×10^8, 5.0×10^8, and 10.0×10^8 of human platelets were 0.6% ± 0.3%, 2.0% ± 1.6%, and 10.3% ± 1.4%, respectively (Figure 1). We used 10.0×10^8 of human platelets for each mouse in this study.

Platelet count and chemical parameters

Blood samples were collected at the time of sacrifice. Platelet count was measured, and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin (Alb), and total cholesterol (T-CHO) were measured and compared between the PBS group and the hPLT group (Fuji Drichem; Fuji Film Inc, Tokyo, Japan) ($n = 8$).

Histological examination

Liver samples were fixed in 10% buffered formalin, and stained with picrosirius red solution, and the liver fibrotic area was quantified using the winROOF visual system (Mitani Co., Tokyo, Japan) ($n = 8$). In addition, specimens were immunostained with an anti-α-smooth muscle actin (SMA) antibody (Dako) and counterstained with hematoxylin. α-SMA expression was also quantified using the winROOF visual system (Japan) ($n = 6$). To assess the hepatocellular mitotic index, liver sections were stained with hematoxylin and eosin, and the number of hepatocytes undergoing mitosis was calculated. In addition, proliferating cell nuclear antigen (PCNA) staining was conducted using a PCNA staining kit (Invitrogen Co., Carlsbad, CA, United States). PCNA-positive hepatocytes and hepatocytes undergoing mitosis were counted in four randomly selected high-power fields (× 200). Liver sections were also incubated with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) antibody (Promega KK, Tokyo, Japan). TUNEL-positive hepatocytes were counted in four randomly selected high-power fields (× 200) on each slide, and calculated as TUNEL-positive hepatocytes/total hepatocytes ($n = 6$).

Hepatocyte growth factor and TGF-β levels in the liver tissue

An enzyme-linked immunosorbent assay (ELISA) kit was used to measure mouse hepatocyte growth factor (HGF) (Institute of Immunology Co., LTD, Tokyo, Japan) and mouse TGF-β (R and D Systems, Minneapolis, MN, United States). ELISAs were used to measure levels of these proteins in 10% liver tissues lysates ($n = 8$).

Detection of liver hydroxyproline content

Hydroxyproline content was determined as described previously[13]. Briefly, 50 mg liver samples were hydrolyzed in 6 mol/L HCl at 120 ℃ for 16 h. After centrifugation, the supernatant was removed and neutralized with 6 mol/L NaOH. The solution was oxidized with Chloramine T (Sigma-Aldrich Corp., St Louis, MO, United States) in acetate/citrate buffer, followed by the addition of Ehrlich's solution (p-dimethylamino-benzaldehyde in 60% HCl4 with isopropanol). The final mixture was incubated at 60 ℃ for 30 min and then at room temperature for 10 min. Absorbance was determined at 560 nm. The value of the hepatic hydroxyproline concentration was expressed as μg/g wet tissue.

α-SMA and MMP-9 expression levels, and signal transduction cascades

For Western blotting analysis, protein was obtained from liver tissues lysates, separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, United States). We used primary antibodies specific for α-SMA (Dako), MMP-9 (AB1916) (Chemicon International, Temecula, CA, United States), phosphoserine mesenchymal-epithelial transition factor (Met) (3127), Met (3135S), phosphotyrosine SMAD3 (9529S), SMAD3 (9513), caspase-3 (9662), cleaved caspase-3 (9962), Bcl-2 (2876), glyceraldehyde-3-phosphate dehydrogenase

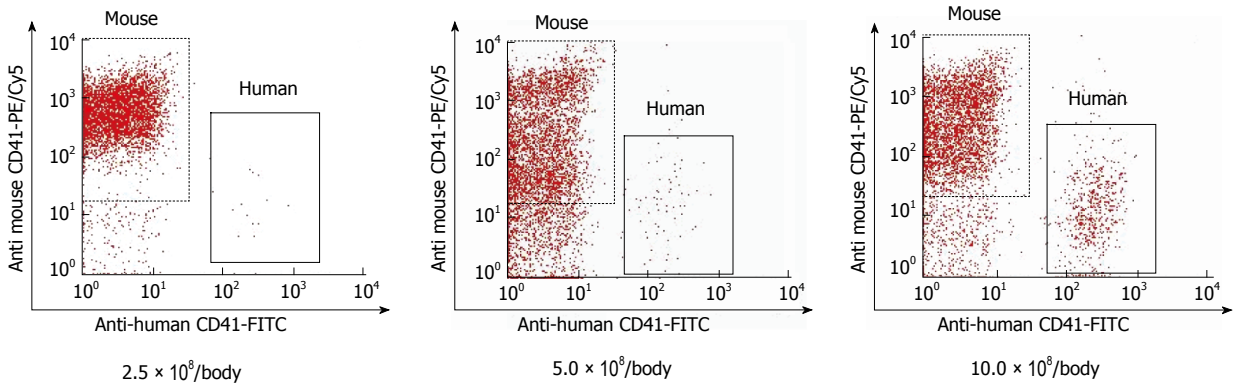

Figure 1 Transfusion conditions. The post-transfusion percentages of human platelets in naïve mice receiving 2.5 × 10^8, 5.0 × 10^8, and 10.0 × 10^8 human platelets. The post-transfusion percentage of human platelets was defined as human platelets/(human platelets + murine platelets). The post-transfusion percentages of human platelets in mice receiving 2.5 × 10^8, 5.0 × 10^8, and 10.0 × 10^8 of human platelets were 0.6% ± 0.3%, 2.0% ± 1.6%, and 10.3% ± 1.4%, respectively. *n* = 3 per group. Data are expressed as the mean ± SD. CD41-FITC: Cluster of differentiation 41-fluorescein isothiocyanate.

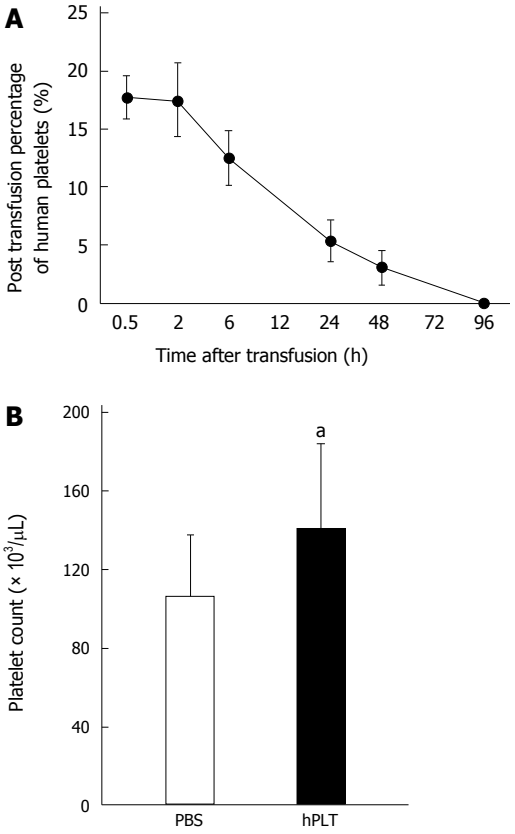

Figure 2 Post-transfusion percentages of human platelets and peripheral platelet counts. A: The post-transfusion percentages of human platelets. Human platelets disappeared from the circulation at 96 h post-transfusion. *n* = 3 per group. Data are expressed as the mean ± SD; B: Peripheral platelet counts. The peripheral platelet count was significantly higher in the human platelet transfusions group than in the phosphate-buffered saline group. *n* = 8 per group. Data are expressed as the mean ± SD. $^aP < 0.05$ using an unpaired *t*-test. PBS: Phosphate-buffered saline; hPLT: Human platelet transfusions.

(GAPDH) (2118), and β-actin (4970) (Cell Signaling Technology, Beverly, MA, United States) and secondary mouse or rabbit antibodies conjugated with horseradish peroxidase (Invitrogen Co.). Immunoblots were analyzed using an enhanced chemiluminescence system. Protein band densities were quantified using densitometry. Band intensities were normalized to those of GAPDH, caspase-3, Met, or SMAD3 (*n* = 3).

Immunohistochemistry for human platelets

Human platelets were transfused to SCID mice with normal or fibrotic livers, and accumulation of the transfused human platelets in the liver 2 h after transfusion was measured and compared between the two groups.

Immunofluorescence staining was performed on 5 μm thick sections of tissue that had been fixed in 4% paraformaldehyde, immersed in OCT compound, and incubated with FITC-conjugated anti-human CD41 antibody (Dako). Stained sections were examined under a confocal laser-scanning microscope (BZ-9000, Keyence Co., Tokyo, Japan).

Statistical analysis

All data are expressed as means ± SD. Unpaired *t*-tests were used to compare two groups. *P* values < 0.05 were considered significant.

RESULTS

The post-transfusion ratio of human platelets and peripheral platelet counts

Human platelets disappeared from the peripheral blood 96 h after transfusion (Figure 2A). The peripheral platelet counts at the time of sacrifice, *i.e.*, 96 h after transfusion, were significantly higher in the hPLT group than in the PBS group ($P < 0.05$) (Figure 2B).

Liver/body weight ratio, PCNA labeling index, mitotic index, and spleen/body weight ratio

There were no significant differences in the liver/body weight ratio, PCNA index, mitotic index, and spleen/body weight ratio between the hPLT and PBS groups (Table 1).

Serum AST, ALT, T-bil, Alb, and T-CHO concentrations

There were no significant differences in the serum AST,

Table 1 Liver regeneration indices and spleen/body weight ratios, serum asparatate aminotransferase, alanine aminotransferase, total bilirubin, albumin, and total cholesterol concentrations

	Liver/body weight ratio	PCNA labeling index (/HPF)	Mitotic index (/HPF)	Spleen/body weight ratio	AST (U/mL)	ALT (U/mL)	T-bil (mg/mL)	Alb (g/mL)	T-CHO (g/mL)
PBS	6.2% ± 0.5%	2.1 ± 0.9	0.6 ± 0.3	0.23% ± 0.04%	50 ± 21	122 ± 56	1.0 ± 0.2	3.0 ± 1.0	77.8 ± 5.4
hPLT	6.7% ± 0.5%	2.4 ± 0.9	0.6 ± 0.5	0.24% ± 0.05%	50 ± 15	104 ± 64	0.8 ± 0.2	3.0 ± 1.5	82.7 ± 4.4[a]

n = 8 per group. [a]P < 0.05 for the human platelet transfusions (hPLT) group *vs* the phosphate-buffered saline (PBS) group. PCNA: Proliferating cell antigen; HPF: High-power field; AST: Asparatate aminotransferase; ALT: Alanine aminotransferase; T-bil: Total bilirubin; Alb: Albumin; T-CHO: Total cholesterol.

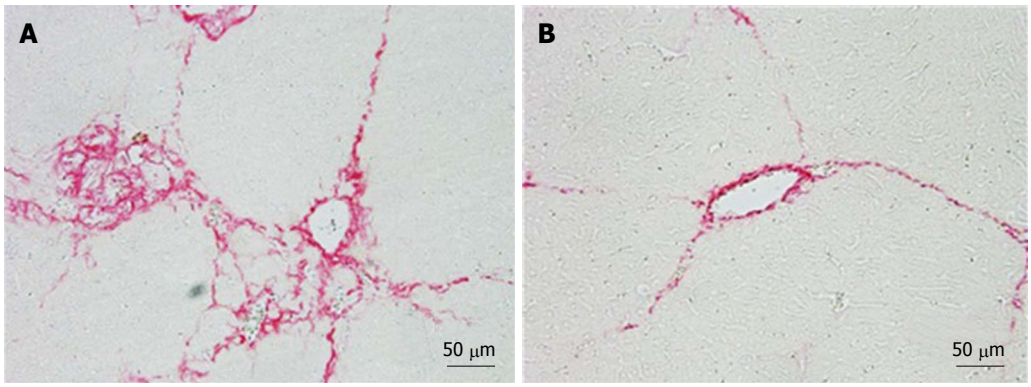

Figure 3 Fibrotic index and hydroxyproline contents. A: The fibrotic index, which was calculated based on the area stained by picrosirius red solution, was significantly lower in the human platelet transfusions (hPLT group) group than in the phosphate-buffered saline (PBS) group; B: The hydroxyproline content in the liver tissue was significantly lower in the hPLT group than in the PBS group. n = 8 per group.

T-bil, and Alb levels between the PBS and hPLT groups. Despite the lack of statistically significant differences, there was a tendency for the serum ALT level to be lower in the hPLT group than in the PBS group (P = 0.3). The serum T-CHO level was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Table 1).

Fibrotic index and liver hydroxyproline content

The fibrotic index, which was calculated based on the area stained with picrosirius red solution, was significantly lower in the hPLT group than in the PBS group (P < 0.05) (Figure 3A). In addition, the liver hydroxyproline content was significantly lower in the hPLT group than in the PBS group (P < 0.05) (Figure 3B).

α-SMA and TUNEL stainings and MMP-9, Bcl-2, caspase-3, and cleaved caspase-3 expression levels

There was less α-SMA staining in the hPLT group compared to the PBS group (Figure 4A and B). TUNEL staining revealed only a few apoptotic cells in the hPLT group, whereas several apoptotic hepatocytes were observed in the PBS group (Figure 4C and D). α-SMA expression calculated based on the area stained by anti-α-SMA antibody and TUNEL positive hepatocytes/total hepatocytes were significantly lower in the hPLT group than in the PBS group (both P < 0.05) (Figure 4E).

MMP-9 expression was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Figure 4F and G). Cleaved caspase-3 expression was significantly lower in the hPLT group than in the PBS group (P < 0.05), whereas Bcl-2 was more robustly expressed in the hPLT group as compared to the PBS group (P < 0.01) (Figure 4F and G).

Mouse HGF and TGF-β levels in the liver tissues and cellular signal transduction

Expression of mouse HGF in the liver tissue was significantly higher in the hPLT group than in the PBS group (P < 0.05) (Figure 5A). The concentration of mouse TGF-β was significantly lower in the liver tissues of the hPLT group than in the PBS group (P < 0.05) (Figure 5B).

There was increased Met phosphorylation in the hPLT group compared to the PBS group (P < 0.05) (Figure 5C and D). Although the difference was not statistically significant, SMAD3 phosphorylation was lower in the hPLT group than in the PBS group (P = 0.1) (Figure 5C and D).

Accumulation of human platelets in the liver

Significant human platelet accumulation in the liver was observed in the fibrotic liver tissues, whereas fewer platelets accumulated in the normal liver (Figure 6).

DISCUSSION

We demonstrated that human platelets suppressed liver fibrosis in SCID mice. It was suspected that these anti-fibrotic effects were due to an increased concentration of HGF in the liver, resulting in decreased TGF-β concentrations and increased MMP-9 levels. Furthermore, inhibition of hepatocyte apoptosis by HGF may have suppressed

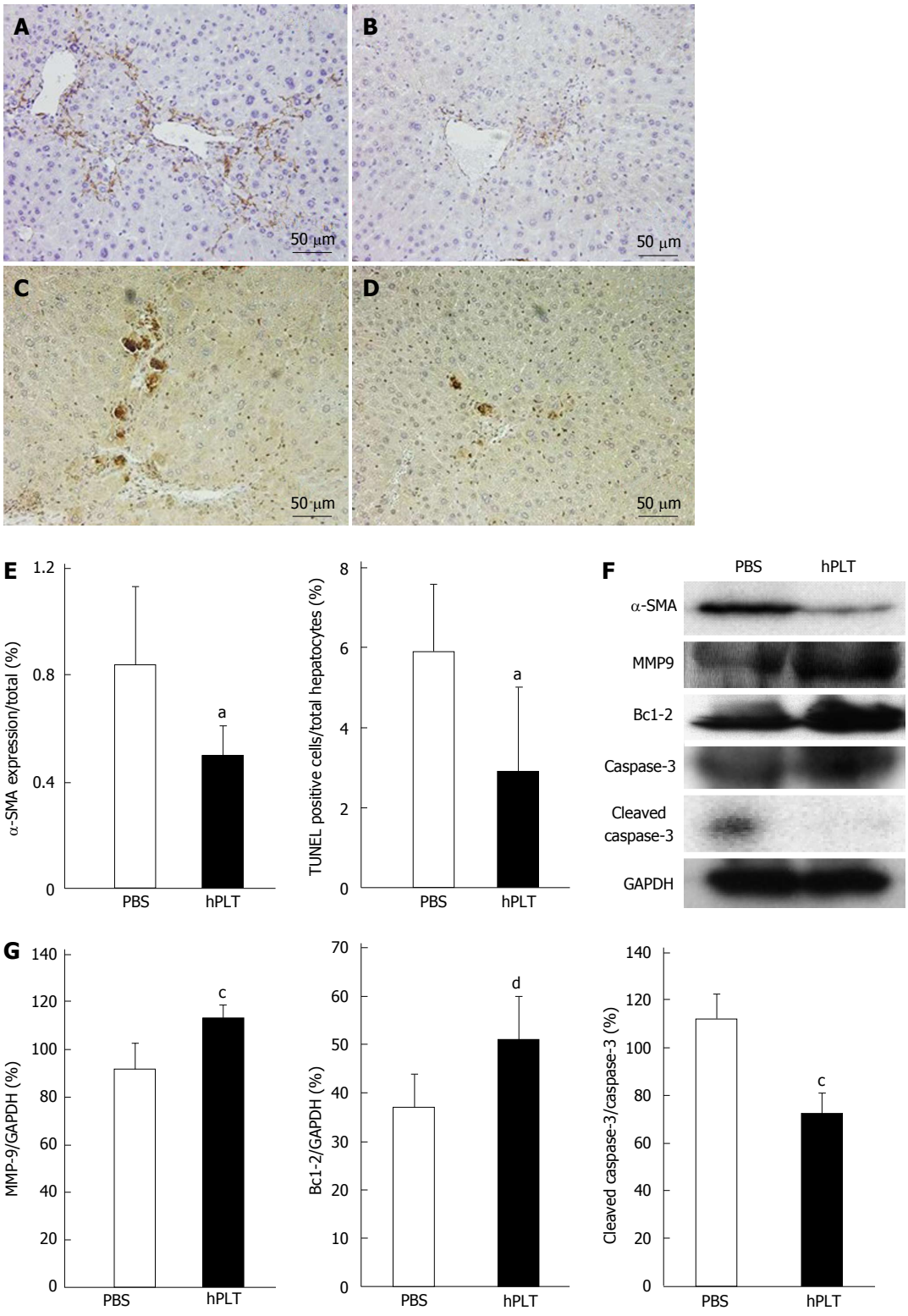

Figure 4 α-smooth muscle actin and TUNEL staining. Matrix metalloproteinase-9 (MMP-9), Bcl-2, caspase-3, cleaved caspase-3 expression levels. A, B: Immunostaining of α-smooth muscle actin (α-SMA) in the phosphate-buffered saline (PBS group) or human platelet transfusions (hPLT group). The α-SMA staining was less robust in the hPLT group than in the PBS group; C, D: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the PBS and hPLT groups. Few apoptotic cells were observed in the hPLT group, whereas several apoptotic hepatocytes were observed in the PBS group; E: α-SMA expression calculated based on the area stained by anti-α-SMA antibody and TUNEL-positive hepatocytes/total hepatocytes in the PBS and hPLT groups. n = 6 per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired t-test. α-SMA expression and the number of apoptotic hepatocytes in the hPLT group were lower than those in the PBS group; F: α-SMA, MMP-9, Bcl-2, caspase-3, and cleaved caspase-3 expression levels assessed with Western blotting. α-SMA and cleaved caspase-3 expression levels were less intense in the hPLT group than in the PBS group, whereas MMP-9 and Bcl-2 expression levels were stronger in the hPLT group than in the PBS group; G: MMP-9, Bcl-2, and cleaved caspase-3 expression levels were quantified using densitometry. n = 3 per group. Data are expressed as the means ± SD. $^{c}P < 0.05$ and $^{d}P < 0.01$ using an unpaired t-test. MMP-9 and Bcl-2 expression levels were significantly higher in the hPLT group than in the PBS group, whereas cleaved caspase-3 expression was significantly lower in the hPLT group than in the PBS group. Bcl-2: B-cell lymphoma-2.

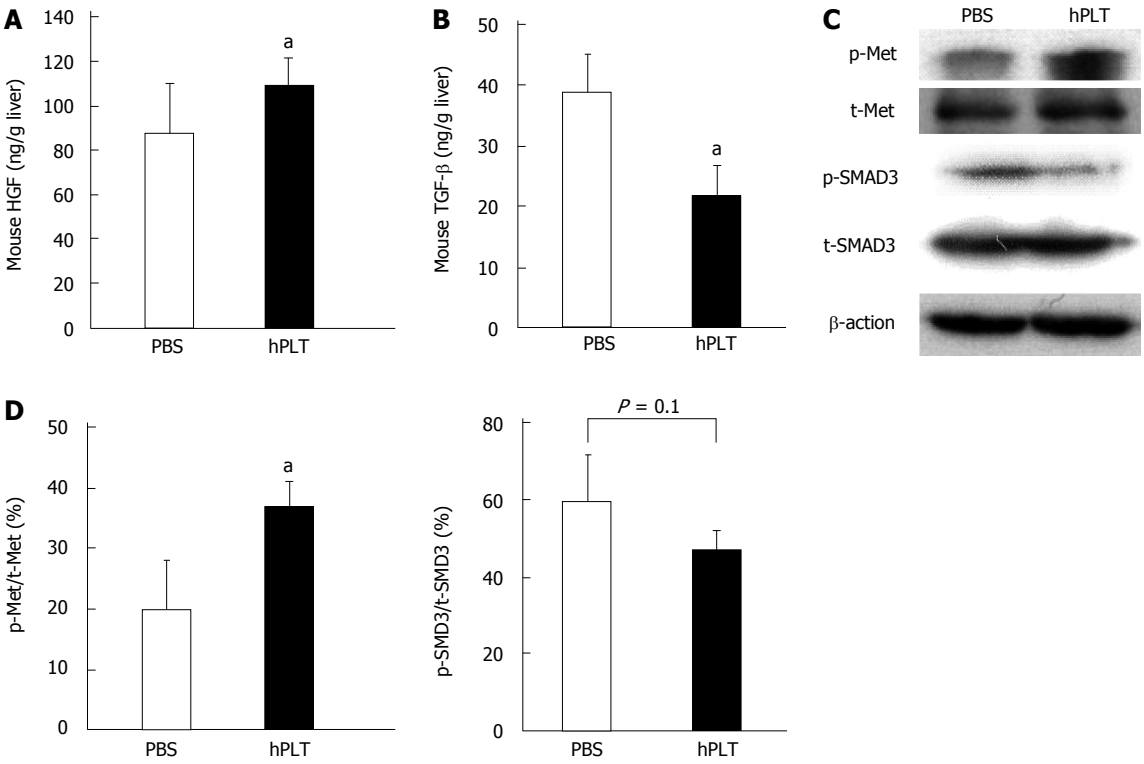

Figure 5 Mouse hepatocyte growth factor and transforming growth factor-β in liver tissue and cellular signal transductions. A: Mouse hepatocyte growth factor (HGF) concentrations in liver tissue. $n = 8$ per group. Data are expressed as the means ± SD. $^{a}P < 0.05$ for the human platelet transfusions (hPLT) group *vs* the phosphate-buffered saline (PBS) group using an unpaired *t*-test. Mouse HGF expression was significantly higher in the hPLT group than in the PBS group; B: Mouse transforming growth factor-β (TGF-β) concentrations in liver tissue. $n = 8$ per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired *t*-test. Mouse TGF-β expression was significantly lower in the hPLT group than in the PBS group; C: Phosphorylation of mesenchymal-epithelial transition factor (Met) and SMAD3 in the PBS and hPLT groups. Met was more highly phosphorylated in the hPLT group than in the PBS group, whereas phosphorylation of SMAD3 was weaker in the hPLT group than in the PBS group; D: Met and SMAD3 phosphorylation levels were quantified using densitometry. $n = 3$ per group. Data are expressed as the mean ± SD. $^{a}P < 0.05$ using an unpaired *t*-test. Phosphorylation of Met was significantly higher in the hPLT group than in the PBS group. Although the difference was not statistically significant, phosphorylation of SMAD3 tended to be lower in the hPLT group than in the PBS group.

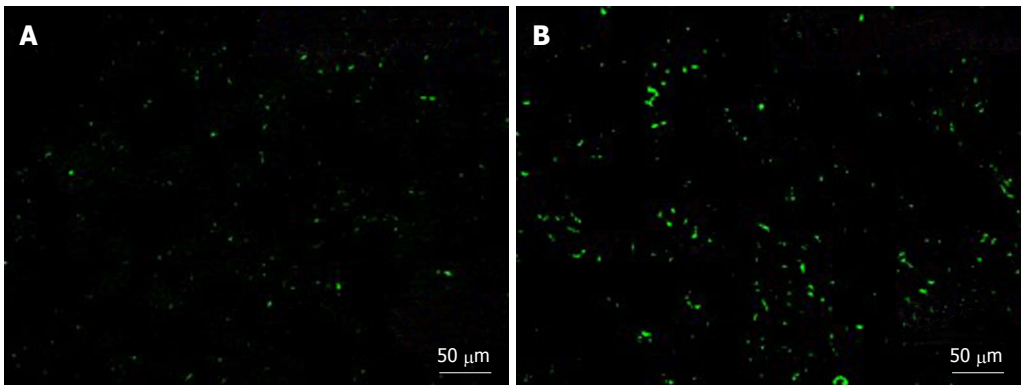

Figure 6 Accumulation of transfused human platelets in the liver. A: Normal liver; B: Fibrotic liver. Immunostaining images obtained using anti-human CD41 antibody 2 h after transfusion. Significant human platelet accumulation in the liver was observed in the fibrotic liver, whereas few platelets accumulated in the normal liver.

HSC activation, resulting in decreased fibrotic changes. These results, together with recent reports showing that platelets contribute to liver regeneration[12,14-21], suggest that platelet increment therapy, such as thrombopoietin administration and platelet transfusions, may provide new clinical approaches for the treatment of liver diseases.

Platelets contain three types of secretory granules, notably α-granules, dense-granules, and lysosomal granules[22]. Each granule contains growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), HGF, vascular endothelial growth factor, serotonin, ATP, and epidermal growth factor, among others[22]. The granule constituents of platelets exhibit species differences, *i.e.*, although rodent platelets contain a large amount of HGF[16,23], human platelets

do not[24]. Platelets accumulate in the liver in response to various conditions, such as ischemia and reperfusion[25], cirrhosis[26], cholestasis[27], and viral hepatitis[28]. Although most studies have evaluated platelets as promoters of inflammatory responses and liver injury[25,26,28], recent scientific[12,14-19] and clinical data[20,21] have revealed additional and different roles for platelets in the liver. We previously showed that platelets accelerate liver regeneration through three different mechanisms: a direct effect on hepatocytes[14,16], a cooperative effect with liver sinusoidal endothelial cells[18], and a collaborative effect with Kupffer cells[12]. Furthermore, platelets are reported to have anti-fibrotic and fibrolytic effects on the liver[7-10]. We have indicated that thrombopoietin-induced thrombocytosis attenuated fibrotic changes in rodents[7,8]. Kodama *et al*[9] reported that platelets exert an anti-fibrotic role by suppressing collagen type I expression *via* the HGF/Met signaling pathway. Ikeda *et al*[10] demonstrated that human platelet-derived ATP suppressed the activation of HSCs through the adenosine-cyclic 5'-adenosine monophosphate signaling pathway. In addition, Maruyama *et al*[29] reported that platelet transfusion once a week for 12 wk decreased serum hyaluronic acid concentrations, a fibrotic marker, in chronic hepatitis patients with Child-Pugh class A or B. In the present study, human platelet transfusion inhibited liver fibrosis in SCID mice. The elevated peripheral platelet counts and the higher serum T-CHO concentrations after transfusion were consequences of reduced liver cirrhosis. Furthermore, the increased number of platelets that accumulated in the fibrotic liver implied that transfused platelets accumulation was induced in the fibrotic liver and released biologically-active substances, such as ATP, which directly suppresses HSC activation and decreases fibrosis[10].

HSCs undergo a complex transformation and activation process during which the cells morphologically change from quiescent oval-shaped cells to activated spindle-shaped cells. The activation of HSCs correlates with α-SMA expression[30]. TGF-β is produced by HSCs and Kupffer cells and is recognized as the main pro-fibrogenic mediator that triggers HSC activation. Hepatic TGF-β concentrations have been shown to be increased among patients with liver cirrhosis[31]. The effects of TGF-β are mediated by intracellular signaling via SMAD proteins, which modulate the transcription of target genes[32]. Following ligand binding to the TGF-β type Ⅱ receptors, the TGF-β type Ⅰ receptor becomes activated. SMAD3 proteins associate with the activated receptor and become phosphorylated, allowing the formation of oligomeric complexes with SMAD4. This heterotrimeric complex translocates into the nucleus and binds to specific nucleotide motifs to regulate transcription of target genes such as *COL1A2*, which encodes the collagen α-2 (1) chain in HSCs[32]. In the present study, although there were no significant differences in the liver/body weight ratio, spleen/body ratio, and liver regeneration indexes, fibrogenic markers such as the fibrotic index, hydroxyproline content, and expression of α-SMA were decreased upon human platelet transfusion. In addition, TGF-β concentration decreased with subsequent suppression of SMAD3 phosphorylation after platelet transfusion. These results indicated that human platelet transfusion might have suppressed liver fibrosis by reducing the TGF-β concentration in the liver.

HGF is predominantly produced by Kupffer cells[33]. HGF is known for its major roles in liver development and regeneration by exerting mitogenic and morphogenic effects on hepatocytes. After HGF binds to Met, Met is phosphorylated and intracellular adapter proteins activate distinct intracellular signals, such as the PI3K, Ras, and ERK pathways, and execute pro-mitogenic and anti-apoptotic functions[34]. HGF contributes to the resolution of fibrosis by regulating TGF-β and MMP levels[35]. Giebeler *et al*[36] reported that hepatocyte-specific Met knockout mice exhibited increased expression of TGF-β, α-SMA, and collagen-1α messenger RNA, and enhanced collagen fiber staining. Kanemura *et al*[37] reported that up-regulated HGF expression after human *HGF* gene delivery induced higher MMP activities. In the present study, the mouse HGF concentration in the liver tissue was elevated after human platelet transfusion. Because human platelets do not contain significant amounts of HGF[24], it was suspected that the expression of HGF in the liver might be elevated because of enhanced release from Kupffer cells or an increased amount of mouse platelet accumulation in the liver, leading to a reduction in the TGF-β concentration and attenuated HSC activation. Furthermore, HGF might have enhanced the production of MMP-9, which promotes fibrinolysis in the liver.

In recent years, liver fibrosis has been considered to be associated with hepatocyte apoptosis[4]. Hepatic fibrosis was shown to be significantly reduced when Fas-mediated apoptosis was impaired or when caspases were inhibited[38]. Moreover, persistent hepatocyte apoptosis has been shown to lead to liver fibrosis due to hepatocyte disruption of Bcl-xL[39]. Engulfment of apoptotic bodies by Kupffer cells has been demonstrated to promote TGF-β production, and phagocytosis of apoptotic bodies by HSCs leads to their activation and increased production of TGF-β and collagen type Ⅰ. Hisakura *et al*[40] reported that platelets protect against hepatocyte apoptosis and induce immediate activation of the Akt pathway, followed by an increase in Bcl-xL and a decrease in cleaved caspase-3 in hepatocytes. In the present study, hepatocyte apoptosis and expression of cleaved caspase-3 were suppressed and Bcl-2, an inhibitor of caspase-3, was increased by human platelet transfusion. It was hypothesized that inhibition of apoptosis by human platelet transfusion might help suppress liver fibrosis. Specifically, because HGF has an anti-apoptotic effect[34], elevated HGF levels may contribute to the inhabitation of hepatocyte apoptosis.

However, several questions remain. First, there are several types of growth factors in platelets that exert pro-fibrotic or anti-fibrotic effects. For example, platelet-derived chemokine ligand 4[26] and PDGF[41] induce HSC activation, whereas ATP[10] and IGF-1[42] suppress

HSC activation. It is difficult to explain the pro-fibrotic or anti-fibrotic effects by one or two substances within platelets. In addition, there are many cell types in the liver, such as hepatocytes, Kupffer cells, HSCs, and liver sinusoidal endothelial cells, that are involved in liver fibrogenesis. Therefore, it is important to view these results from a comprehensive perspective. Second, in this study, there were no differences in liver regeneration between the PBS and hPLT groups, which differed from our previous study[7]. It has been reported that a higher dose of CCl_4 is necessary to induce liver fibrosis in SCID mice compared to wild-type mice[43]. In this study, the degree of liver fibrosis was reduced compared to the previous study. The reduced fibrosis in the current model may have contributed to the low PCNA labeling index and hepatocyte mitosis in the hPLT group. Furthermore, in our previous study, we induced thrombocytosis using thrombopoietin, which resulted in higher peripheral platelet counts than those observed in this study. These differences in the degree of fibrosis and peripheral platelet counts may underlie the discrepancies in the results related to the requirement for the hepatocyte cell cycle and mitosis. Third, HGF and TGF-β are both produced by Kupffer cells, and the discrepancy in the dynamics of these growth factors was not clear. Because TGF-β is also produced by HSCs, it is possible that the increased HGF levels resulting from human platelet transfusion mainly suppressed HSC activity and down-regulated TGF-β expression in the liver. Fourth, although there was a significant difference in hepatocyte apoptosis as evaluated by TUNEL staining, serum AST and ALT concentrations were not significantly different. In our fibrosis model using CCl_4 with this duration and dose, it was difficult to induce strong fibrosis and apoptosis of hepatocytes in SCID mice. Despite statistically significant differences in the number of apoptotic hepatocytes between the PBS and hPLT groups, the difference was small considering the damage to the entire liver. Therefore, the damage did not reflect the serum AST and ALT concentrations.

ACKNOWLEDGMENTS

The authors thank Dr. Kozuma Y (Department of Medical Science, University of Tsukuba) for his support in flow cytometric analysis and Ms. Takahashi A (Department of Surgery, University of Tsukuba) for her support in Western blotting.

COMMENTS

Background

Liver cirrhosis is the ultimate stage of liver fibrosis, and there are currently no specific treatments that inhibit progressive fibrosis. Hepatocyte growth factor (HGF) helps resolve fibrosis by regulating transforming growth factor-β (TGF-β), matrix metallopeptidases (MMPs), and hepatocyte apoptosis.

Research frontiers

Platelets have been conventionally regarded as an exacerbating factor to inflammatory response and injury in the liver. However, recent studies have demonstrated the role of platelets in promoting liver regeneration, improving liver fibrosis, and attenuating hepatitis. In this study, authors assessed the effects of human platelet transfusion on liver fibrosis.

Innovations and breakthroughs

Platelets contain three types of secretory granules: α-granules, dense-granules, and lysosomal granules. Each granule contains growth factors. The granule constituents of platelets exhibit species differences, *i.e.*, human platelets do not contain significant amounts of HGF. This is the first study to show that human platelets have a role in suppressing liver fibrosis.

Applications

By demonstrating that human platelets suppress liver fibrosis, this study represents a potential future strategy for platelet therapy in the treatment of patients with liver cirrhosis.

Terminology

HGF is known for its major roles in liver development and regeneration. After HGF binds to mesenchymal-epithelial transition factor (Met), Met is phosphorylated, and intracellular adapter proteins activate distinct intracellular signals, and execute pro-mitogenic and anti-apoptotic functions. HGF is known to contribute to the resolution of fibrosis by regulating TGF-β and MMP levels.

Peer review

The authors examined the role of human platelets on liver fibrosis. It was revealed that increased concentrations of HGF in the liver suppressed hepatic stellate cell activation, induced MMPs, and inhibited hepatocyte apoptosis. The results are interesting and may provide new clinical approaches for the treatment of liver cirrhosis.

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P- Reviewers Mohanakumar T, Theret N
S- Editor Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5261

World J Gastroenterol 2013 August 28; 19(32): 5261-5270
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Human development index is associated with mortality-to-incidence ratios of gastrointestinal cancers

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Author contributions: Hu QD and Liang TB conceived the study; Hu QD and Zhang Q, Liang TB designed the study; Hu QD collected data; Hu QD, Zhang Q, Chen W and Bai XL analyzed the data; Hu QD, Zhang Q and Chen W drafted the manuscript; Liang TB finalized the manuscript, and took responsibility for the integrity of the data and the accuracy of the data analysis; all authors have read and approved the final manuscript.

Supported by The National Natural Science Funds for Distinguished Young Scholars, No. 30925033; and the Innovation and High-Level Talent Training Program of Department of Health of Zhejiang Province, China

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Received: May 18, 2013 Revised: July 7, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

AIM: To identify the role of human development in the incidence and mortality rates of gastrointestinal cancers worldwide.

METHODS: The age-standardized incidence and mortality rates for gastrointestinal cancers, including cancers of the esophagus, stomach, pancreas, liver, gallbladder, and colorectum, were obtained from the GLOBOCAN 2008 database and United States Cancer Statistics (USCS) report. The human development index (HDI) data were calculated according to the 2011 Human Development Report. We estimated the mortality-to-incidence ratios (MIRs) at the regional and national levels, and explored the association of the MIR with development levels as measured by the HDI using a modified "drug dose to inhibition response" model. Furthermore, countries were divided into four groups according to the HDI distribution, and the MIRs of the four HDI groups were compared by one-way ANOVA followed by the Tukey-Kramer *post-hoc* test. State-specific MIRs in the United States were predicted from the estimated HDI using the fitted non-linear model, and were compared with the actual MIRs calculated from data in the USCS report.

RESULTS: The worldwide incidence and mortality rates of gastrointestinal cancers were as high as 39.4 and 54.9 cases per 100000 individuals, respectively. Linear and non-linear regression analyses revealed an inverse correlation between the MIR of gastrointestinal cancers and the HDI at the regional and national levels ($\beta < 0$; $P = 0.0028$ for regional level and < 0.0001 for national level, ANOVA). The MIR differed significantly among the four HDI areas (very high HDI, 0.620 ± 0.033; high HDI, 0.807 ± 0.018; medium HDI, 0.857 ± 0.021; low HDI, 0.953 ± 0.011; $P < 0.001$, one-way ANOVA). Prediction of the MIRs for individual United States states using best-fitted non-linear models showed little deviation from the actual MIRs in the United States. Except for 28 data points (9.93% of 282), the actual MIRs of all gastrointestinal cancers were mostly located in the prediction intervals *via* the best-fit non-linear regression models.

CONCLUSION: The inverse correlation between HDI and MIR demonstrates that more developed areas have a relatively efficacious healthcare system, resulting in low MIRs, and HDI can be used to estimate the MIR.

Key words: Gastrointestinal neoplasms; Mortality-to-incidence ratio; Human development index; Healthcare disparities; Socioeconomic factors

Core tip: This study is the first to explore the exact re-

lationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. We showed the association between the mortality-to-incidence ratios (MIRs) and the human development index at the regional and national levels using a modified "drug dose to inhibition response" model. Further prediction of the MIRs for individual United States states on the basis of best-fitted non-linear models showed little deviation from the actual MIRs in the United States.

Hu QD, Zhang Q, Chen W, Bai XL, Liang TB. Human development index is associated with mortality-to-incidence ratios of gastrointestinal cancers. *World J Gastroenterol* 2013; 19(32): 5261-5270 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5261.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5261

INTRODUCTION

The digestive system includes multiple organs within or alongside the alimentary tract and is of vital importance in the proper functioning of the body. Currently, gastrointestinal cancer is a leading cause of cancer-related deaths in many developed countries, and it has been predicted to have the highest incidence and mortality rates worldwide, irrespective of the level of a country's resources[1-3]. Gastrointestinal cancers are known to notably affect the pathophysiological condition and functioning of the digestive system. Both cancer incidence and mortality in highly developed countries such as the United States peaked in the early 1990s and have since declined because of enhanced awareness, preventive measures, earlier detection and the availability of new and more effective treatment regimens, although very little progress has been made in the treatment of some cancers such as pancreatic cancer[4]. In contrast, limited or inaccessible healthcare resources in developing areas remain barriers to the effective control of future changes in incidence and mortality rates[5,6]. The expected cancer burden will continue to be a serious public health problem in the coming decade, particularly in developing countries[3,7,8].

Disparities in healthcare have received considerable attention from international organizations and national governments[9,10]. The socioeconomic determinants of the inequality reflect regional imbalances in human development. A previous study found that 35% of the cancer deaths may be attributable to nine modifiable risk factors: alcohol, smoking, low fruit and vegetable intake, overweight and obesity, physical inactivity, urban air pollution, unsafe sex, contaminated injections in health care settings, and indoor smoke from household activities such as cooking or indoor heating[11]. Most of these risk factors vary widely among populations in areas with different levels of development[12,13]. However, there is little knowledge about the healthcare disparities in the individuals suffering from gastrointestinal cancers. This study is the first to explore the exact relationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. We aimed to identify the role of human development in the incidence and mortality rates of gastrointestinal cancers worldwide.

MATERIALS AND METHODS

Incidence and mortality data

The global incidence and mortality estimates for gastrointestinal cancers in 184 countries were obtained from the GLOBOCAN 2008 database (http://globocan.iarc.fr/) maintained by the WHO International Agency for Research on Cancer[14,15]. GLOBOCAN also provided regional estimates for each continent.

United States Cancer Statistics (USCS) reported the incidence and mortality rates associated with cancers in United States states[16]. State-specific incidence data were collected from the National Program of Cancer Registries and the Surveillance, Epidemiology, and End Results Program. Mortality information was collected by the National Vital Statistics System, National Center for Health Statistics and United States Centers for Disease Control and Prevention (United States-CDC).

We obtained the data of the incidence and mortality rates of gastrointestinal cancers in six major sites, namely, the esophagus, stomach, pancreas, liver, gallbladder, and colorectum. The USCS did not provide gallbladder cancer data. The overall rates of gastrointestinal cancers were estimated by addition of the rates of the six cancers. The rates were age-standardized using the world standard population and a previously proposed method[17], and presented as age-standardized rates (ASR). ASR is a summary measure of the rate that a population distribution would have if it had a standard age structure. Since age has a powerful influence on the risk of cancer, standardization is necessary when comparing several populations that differ with respect to age[14].

Mortality-to-incidence ratio

The mortality-to-incidence ratios (MIRs) were calculated from the obtained incidence and mortality rates provided by the GLOBOCAN database[14] and USCS report[16]. Extreme MIRs (0, 1, or > 1) were considered abnormal because of (1) illogical data (zero mortality or mortality more than incidence); and (2) zero incidence, and these results were excluded from the regression fit and further analysis. Respectively, 25, 13, 62, 82, 46, and 0 extreme MIR results were excluded in the analysis for cancers of the esophagus, stomach, pancreas, liver, gallbladder, and colorectum.

Estimated human development index

The human development index (HDI) data of Union Nation members in 1980-2011 were available in the United Nations Development Programme (UNDP) database (http://hdr.undp.org/en/statistics). The HDI was calculated according to the 2011 Human Development Report (HDR 2011)[18]. The HDI of Taiwan was

obtained from the National Statistics (Taiwan) website (http://www.stat.gov.tw), and subsequently verified.

We further estimated the state-specific HDI in the United States on the basis of data provided by various data agencies. Information on life expectancy at birth provided by the CDC was adapted by the American HDI Project[19]. The gross domestic product (GDP) per capita was acquired from the Bureau of Economic Analysis at the United States Department of Commerce, and compiled by the Bureau of Business and Economic Research, University of New Mexico[20]. The GDP values were converted to international dollars using purchasing power parity rates. The mean duration of education was estimated from the 2009 American Community Survey data provided by the United States Census Bureau[21], according to the method of Barro and Lee[22]. The expected duration of education in the United States was defined as 12 years; this value was adapted from the United Nations Educational, Scientific and Cultural Organization Institute for Statistics[23].

Statistical analysis

Only the countries with both epidemiologic data from the GLOBOCAN database and HDI from the UNDP program were included in the analysis. Taiwan was not excluded because its HDI value was available at the National Statistics (Taiwan) website. The number of countries included in our research was 165. Patterns in the MIR of gastrointestinal cancers with respect to the levels of socioeconomic development were investigated by correlating the MIRs to the corresponding HDIs *via* linear or non-linear regression. Linear regression fit was conducted to determine the existence of correlations. Derivation of the slope parameter β from 0 was defined by ANOVA. Correlation existence referred to the significantly non-zero β value. Non-linear regression fit was based on a modified "drug dose to inhibition response" model using the formula:

$$\mathrm{MIR} = \frac{1}{1+10^{(\mathrm{HDI}_{50}-\mathrm{HDI})\times\mathrm{Slope}}},$$

where "HDI_{50}" was the HDI value at half maximal MIR and "slope" was a parameter that indicated the steepness of the slope. The MIRs of the four HDI groups were compared by one-way ANOVA followed by the Tukey-Kramer *post-hoc* test. A P value of less than 0.05 was considered statistically significant. Statistical analysis and plotting were performed using Prism 5 (GraphPad, San Diego, CA, United States). The geographical map showing MIR was created using the open source software TileMill (a GitHub project maintained by MapBox, Washington, WI, United States), with map data sources from the Natural Earth database rendered by the Mapnik Library.

RESULTS

Global incidence and mortality of gastrointestinal cancers

In 2008, gastrointestinal cancers were estimated to have affected a total of 3878986 individuals and caused 2824985 deaths worldwide. The global mortality and incidence rates were as high as 39.4 and 54.9 cases per 100000 individuals, respectively. Colorectal cancer was the third most common cancer with 1235108 incidences among the 27 cancers included in the GLOBOCAN database, and it was the most common cancer among the six gastrointestinal cancers included in the current study. Other prevalent cancers according to the incidences reported in the database included stomach cancer (ranked 4th, with 988602 incidences), liver cancer (6th, 749744 incidences), esophageal cancer (8th, 481645 incidences), pancreatic cancer (13th, 278684 incidences), and gallbladder cancer (21st, 145203 incidences). However, stomach cancer had the highest mortality rate (26.1%, 737419 deaths) among all gastrointestinal cancers. In terms of the mortality rate, liver cancer (ranked 3rd with 695726 deaths), colorectal cancer (4th, 609051 deaths), esophageal cancer (6th, 406533 deaths), pancreatic cancer (8th, 266669 deaths) and gallbladder cancer (17th, 109587 deaths) contributed to 24.6%, 21.6%, 14.4%, 9.4% and 3.9% of all deaths caused by gastrointestinal cancers, respectively.

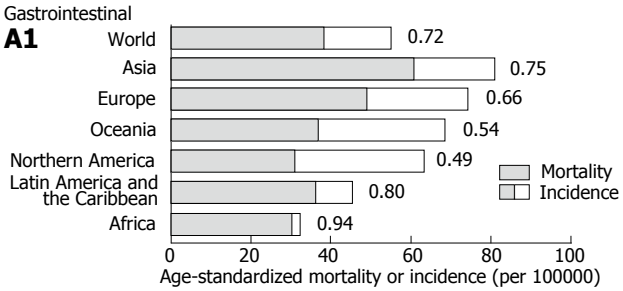
Differences in the regional incidence and mortality

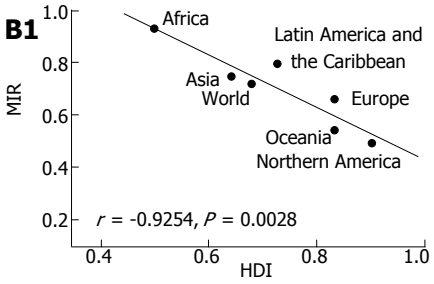
The regional incidence and mortality rates varied among different continents and regions (Figure 1A). Asia had the highest incidence rates of esophageal, stomach and liver cancers, as well as gastrointestinal cancers overall. Interestingly, the MIRs for gastrointestinal cancers, except for pancreatic cancer, were higher in Africa compared with other continents. Linear regression analysis revealed a significant inverse correlation between the regional HDI and MIR of stomach, gallbladder and colorectal cancers and gastrointestinal cancer overall ($P < 0.05$, ANOVA) (Figure 1B).

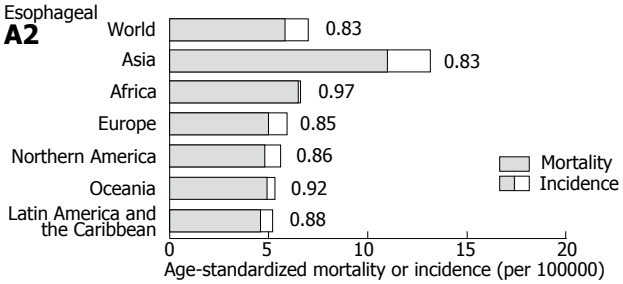
Association between national HDI and MIR

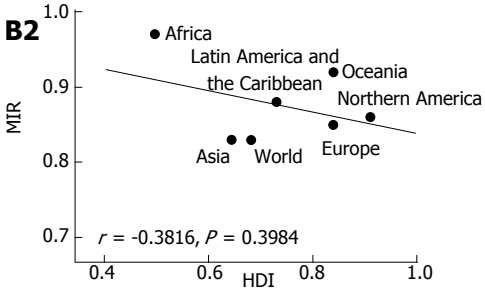
The national MIR varied across different countries with different levels of development, as measured by HDI (Figure 2). Countries with high HDI tended to have relatively low MIR. Cross-national analysis demonstrated that the MIRs of gastrointestinal cancers consistently showed an inverse correlation with the national HDI values *via* linear regression ($\beta < 0$; $P < 0.05$, ANOVA; Table 1, Figure 3A). Furthermore, non-linear regression based on the "drug dose to inhibition response" model was used to analyze this correlation, and a more satisfactory fitting result with larger R square values was achieved for all gastrointestinal cancers (Table 1, Figure 3B). The HDI values at half-maximal MIR (HDI_{50}) in gastrointestinal cancers overall and colorectal cancer were 0.946 and 0.825, respectively. Five other cancers had an HDI_{50} of more than 1.

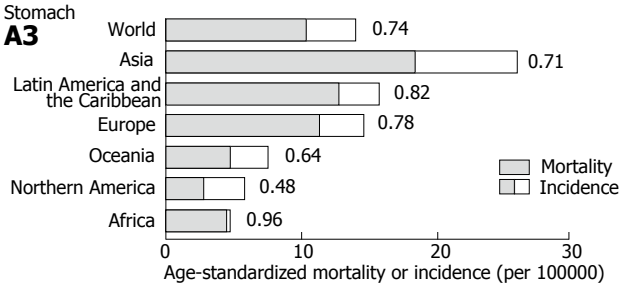
Countries were divided into four groups according to the HDI distribution reported in HDR 2011[18]. The MIR of gastrointestinal cancers differed significantly among these four groups ($P < 0.001$, one-way ANOVA). The mean MIR of countries with very high HDI was

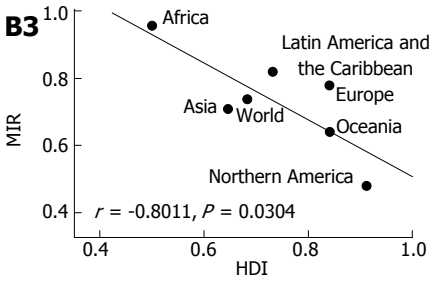

Gastrointestinal
A1
World
Asia
Europe
Oceania
Northern America
Latin America and the Caribbean
Africa
0.72
0.75
0.66
0.54
0.49
0.80
0.94
Mortality
Incidence
0
20
40
60
80
100
Age-standardized mortality or incidence (per 100000)

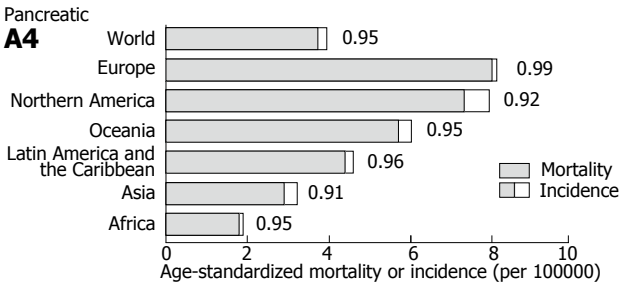

B1
MIR
HDI
Africa
Latin America and the Caribbean
Asia
World
Europe
Oceania
Northern America
r = -0.9254, P = 0.0028

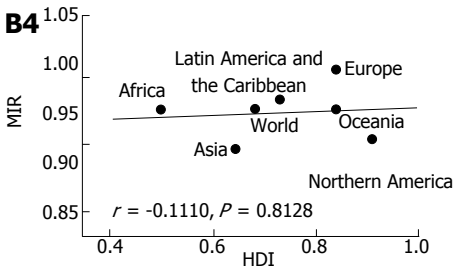

Esophageal
A2
World
Asia
Africa
Europe
Northern America
Oceania
Latin America and the Caribbean
0.83
0.83
0.97
0.85
0.86
0.92
0.88
Mortality
Incidence
0
5
10
15
20
Age-standardized mortality or incidence (per 100000)

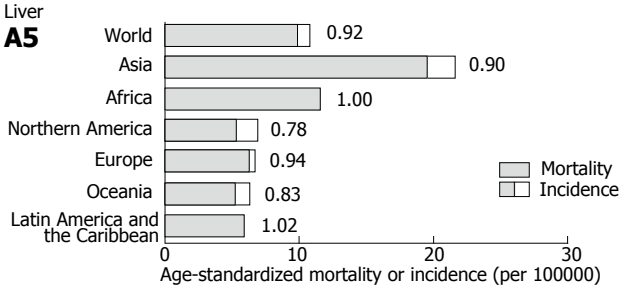

B2
MIR
HDI
Africa
Latin America and the Caribbean
Oceania
Northern America
Asia
World
Europe
r = -0.3816, P = 0.3984

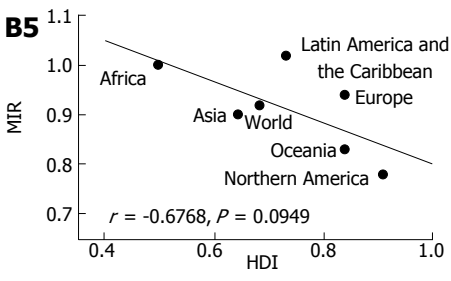

Stomach
A3
World
Asia
Latin America and the Caribbean
Europe
Oceania
Northern America
Africa
0.74
0.71
0.82
0.78
0.64
0.48
0.96
Mortality
Incidence
0
10
20
30
Age-standardized mortality or incidence (per 100000)

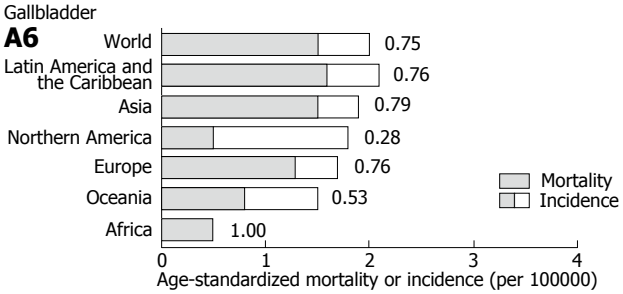

B3
MIR
HDI
Africa
Latin America and the Caribbean
Europe
Asia
World
Oceania
Northern America
r = -0.8011, P = 0.0304

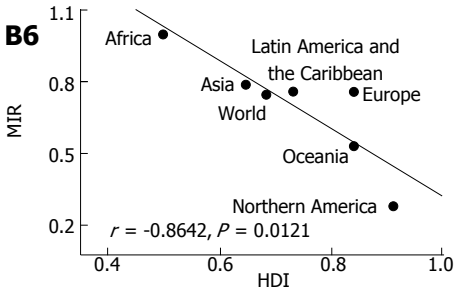

Pancreatic
A4
World
Europe
Northern America
Oceania
Latin America and the Caribbean
Asia
Africa
0.95
0.99
0.92
0.95
0.96
0.91
0.95
Mortality
Incidence
0
2
4
6
8
10
Age-standardized mortality or incidence (per 100000)


B4
MIR
HDI
Latin America and the Caribbean
Europe
Africa
World
Oceania
Asia
Northern America
r = -0.1110, P = 0.8128


Liver
A5
World
Asia
Africa
Northern America
Europe
Oceania
Latin America and the Caribbean
0.92
0.90
1.00
0.78
0.94
0.83
1.02
Mortality
Incidence
0
10
20
30
Age-standardized mortality or incidence (per 100000)


B5
MIR
HDI
Latin America and the Caribbean
Africa
Europe
Asia
World
Oceania
Northern America
r = -0.6768, P = 0.0949


Gallbladder
A6
World
Latin America and the Caribbean
Asia
Northern America
Europe
Oceania
Africa
0.75
0.76
0.79
0.28
0.76
0.53
1.00
Mortality
Incidence
0
1
2
3
4
Age-standardized mortality or incidence (per 100000)


B6
MIR
HDI
Africa
Latin America and the Caribbean
Asia
Europe
World
Oceania
Northern America
r = -0.8642, P = 0.0121

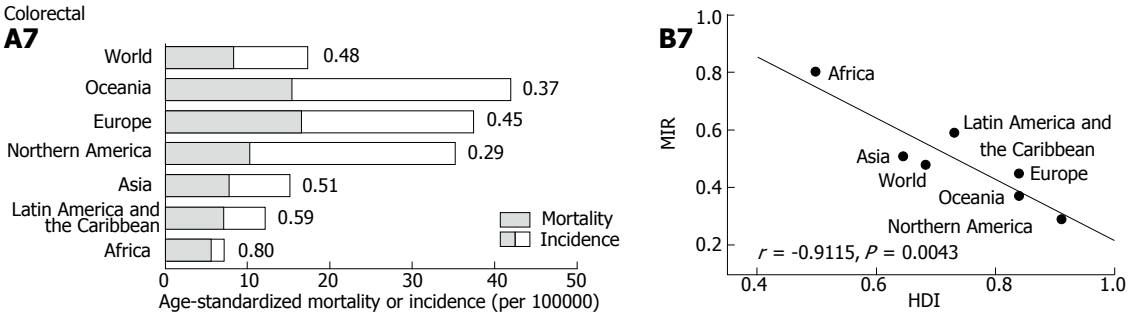


Figure 1 Association between the mortality-to-incidence ratio and human development at the regional level. A: Regional age-standardized mortality (grey) and incidence (white and grey) rates per 100000 individuals for gastrointestinal cancers. The mortality-to-incidence ratios (MIRs) are denoted; B: The regional MIRs of gastrointestinal cancers overall and stomach, liver and colorectal cancers correlate with the human development index (HDI). Best-fit lines by linear regression (solid) are indicated.

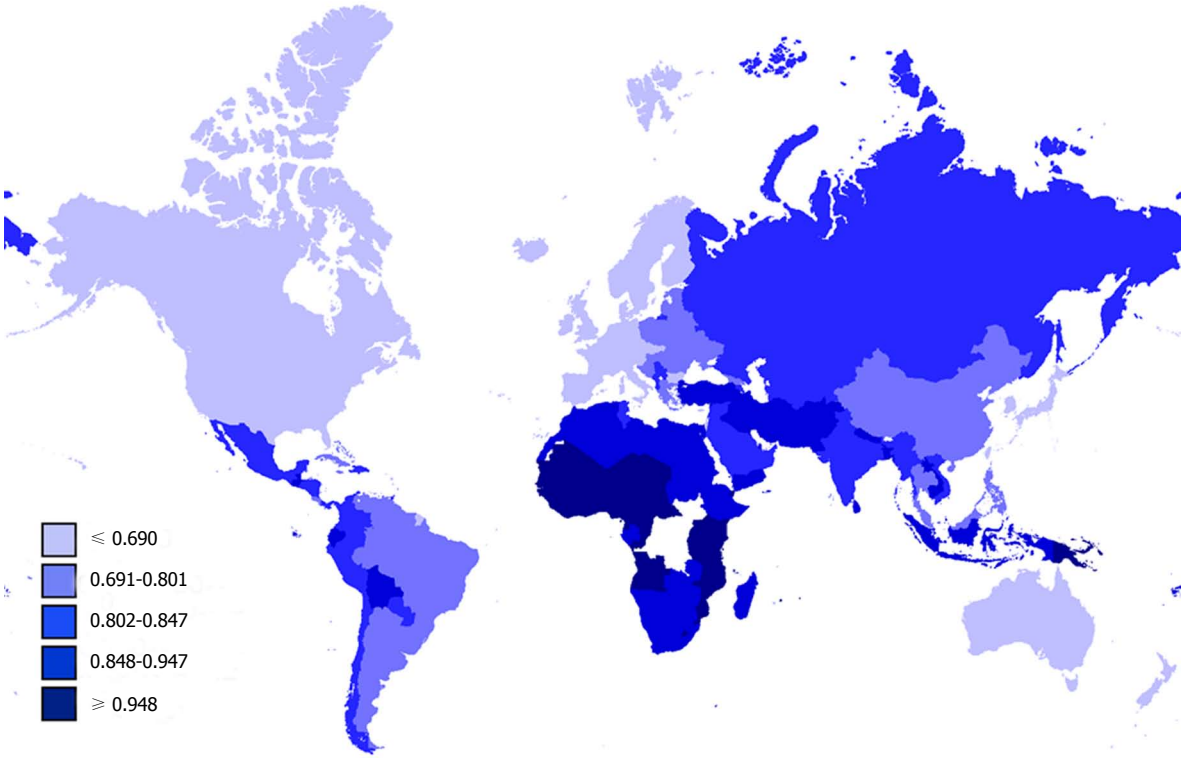


Figure 2 Global mortality-to-incidence ratios of gastrointestinal cancers. Mortality-to-incidence ratios (MIRs) varied across different countries.

0.620 ± 0.033 (95%CI), which was significantly lower than the corresponding values of countries with high, medium, and low HDIs (0.807 ± 0.018, 0.857 ± 0.021, and 0.953 ± 0.011, respectively; $P < 0.05$, Tukey-Kramer *post-hoc* test; Figure 4). Furthermore, there was a significant difference among the four groups in each specific cancer ($P < 0.001$, one-way ANOVA).

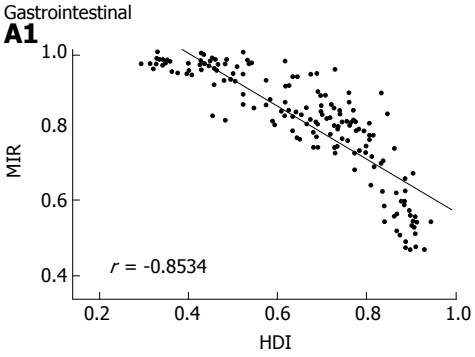
Prediction of MIR in individual United States states

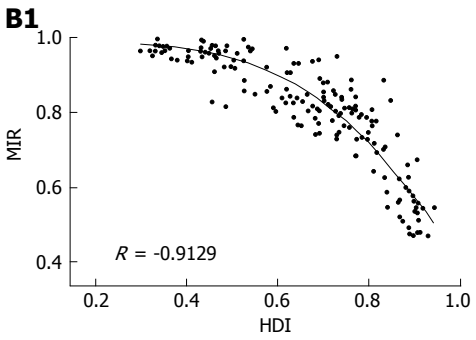
The individual HDIs of 51 United States states were calculated as previously described in HDR 2011. The HDI values in each state ranged from 0.847 to 0.962. To verify the effectiveness of the fitted models, the MIRs of gastrointestinal cancers in each of the United States states were predicted using respective best-fit equations. Except for 28 data points (9.93% of 282), the actual MIRs of all gastrointestinal cancers were mostly located in the prediction intervals *via* the best-fit non-linear regression models. In California, for example, the estimated HDI was 0.907 and the predicted MIR of gastrointestinal cancers was 0.560 ± 0.118 (95% prediction interval, 95%PI). The actual MIR calculated from the reported incidence and mortality was 0.533, and the difference between the actual and predicted MIRs (Δ_{MIR}) was -0.027 (23.1% of 95%PI). The actual MIRs of the six cancers were also in the 95%PI predicted by the respective regression fitting equations (Table 2).

DISCUSSION

Gastrointestinal cancers have high incidence and mor-

Gastrointestinal





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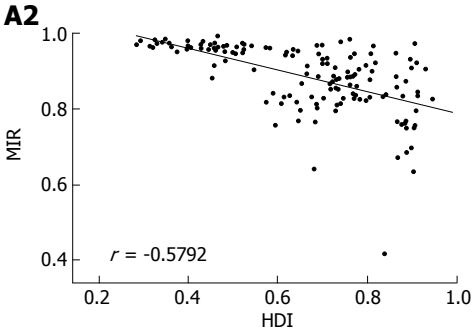

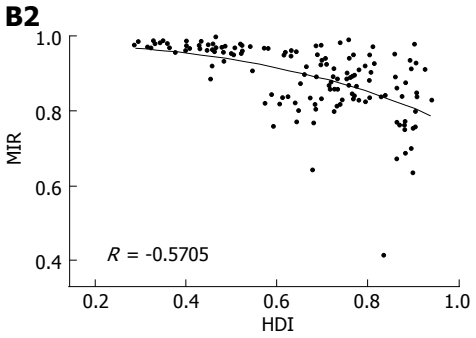

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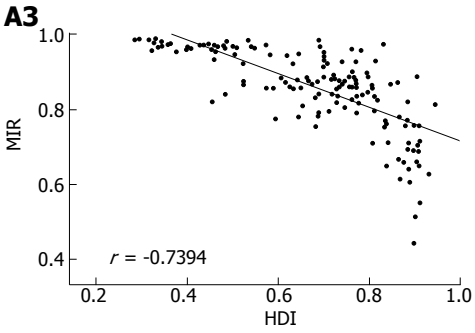
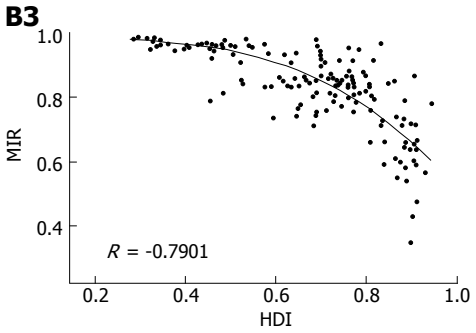
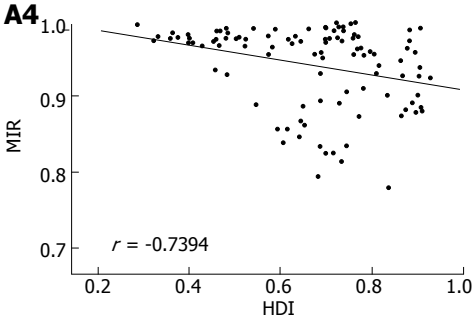
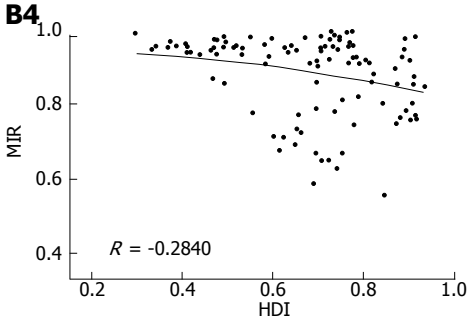


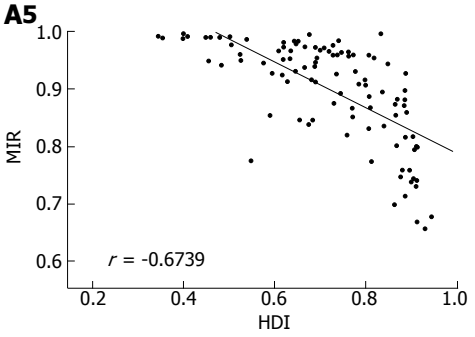


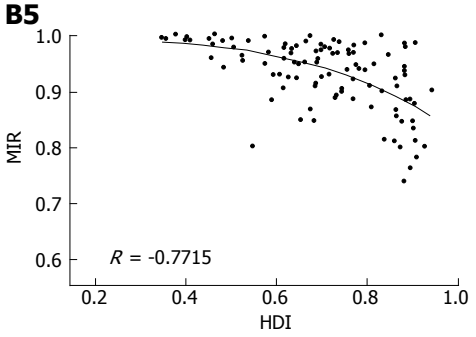
Pancreatic





Liver, gallbladder and pancreatic





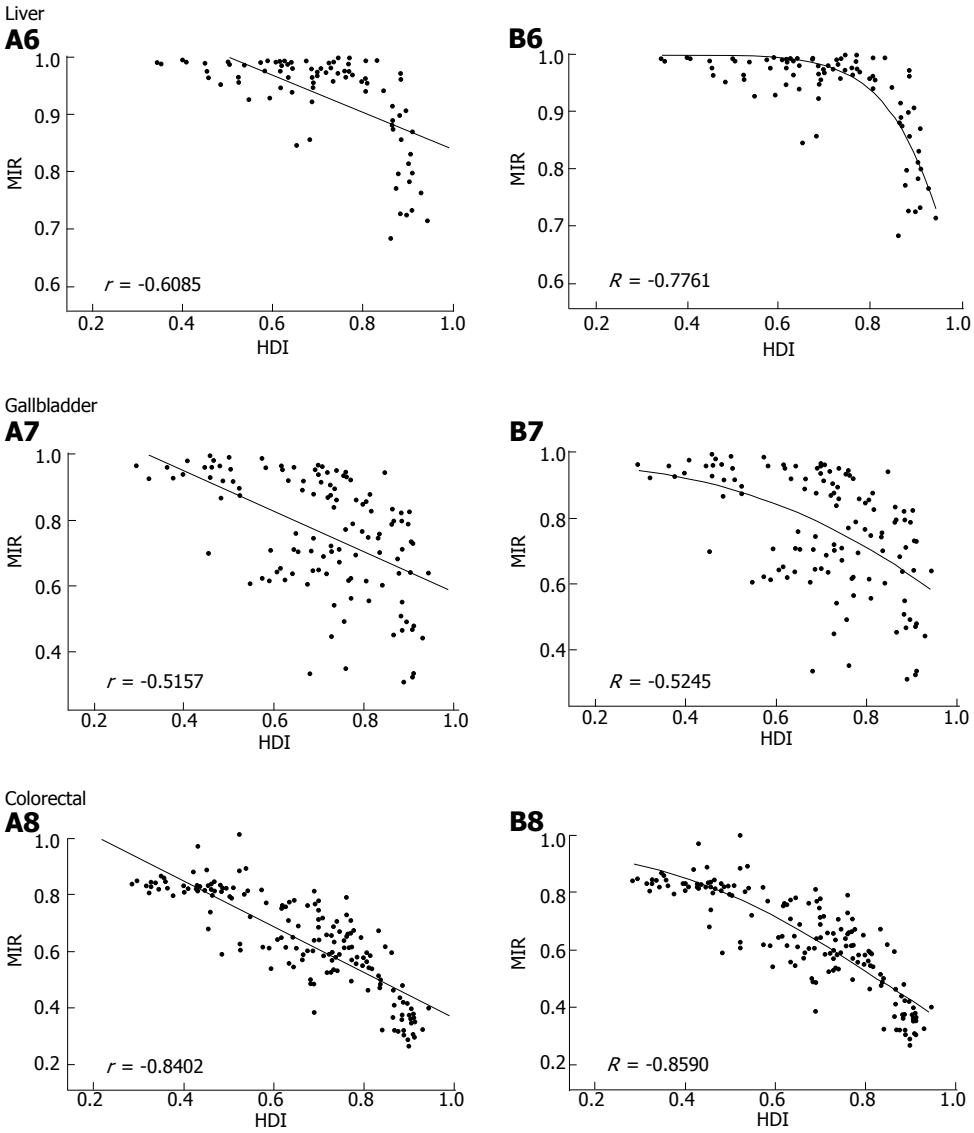


Figure 3 Correlation between national human development index and mortality-to-incidence ratio of gastrointestinal cancers *via* (A) linear or (B) non-linear regression. Best-fit line by regression (solid) is indicated, with *r* or *R* values denoted.

tality rates worldwide[1,24]. We found that both the incidence and mortality rates differed greatly from region to region. Interestingly, the ratio of the mortality rate to the incidence rate, *i.e.*, the MIR, appeared to be higher in less developed regions such as Africa. The development level was quantified by HDI, which is a composite measure of human development. Estimation of national HDI is based on the following parameters: a long and healthy life, access to knowledge, and a decent standard of living[18]. As an indicator of the socioeconomic factor of health, the HDI may serve as the gold standard for international comparisons of development.

MIR is derived as a surrogate indicator of the effectiveness of the health system. It has been proposed as an indirect measure of true biological differences in disease phenotypes or health system-related attributes such as screening, diagnostic modalities, treatment and follow-up[25,26]. An MIR-associated derivative form was identified as a good approximation of the 5-year relative survival for most, but not all, cancers[27]. The MIR is computed from age-standardized rates, and it also reflects a population-based approximation of survival[25]. Accordingly, it could be used to assess the diagnosis proficiency and treatment effectiveness in gastrointestinal cancers.

Africa, which had a relatively low HDI, showed a high MIR for most gastrointestinal cancers, whereas Northern America, which had a higher HDI, showed a low MIR. Furthermore, we found a significant inverse correlation between the regional MIR and corresponding HDIs in some, but not all, gastrointestinal cancers. However, only seven data points were included in the region-specific linear regression analysis. Insufficient sample size for regression analysis might cause fitting inaccuracy[28]. To avoid such inaccuracies, country-specific regression was performed. Linear regression analysis in this study revealed a correlation between the national HDI and MIR in all gastrointestinal cancers. The impact of hu-

Table 1 Results of regression in a cross-national analysis of human development index and mortality-to-incidence ratio

Cancer	Linear regression			Non-linear regression[1]		
	β	*P*	*r*	HDI_{50}	Slope	*R*
All gastrointestinal	-0.703	< 0.001	-0.853	0.946	2.746	-0.9129
Esophageal	-0.295	< 0.001	-0.579	1.362	1.344	-0.5705
Stomach	-0.536	< 0.001	-0.739	1.023	2.372	-0.7901
Pancreatic	-0.097	0.0019	-0.301	2.391	0.706	-0.2840
Liver	-0.322	< 0.001	-0.609	1.026	5.247	-0.7761
Gallbladder	-0.611	< 0.001	-0.516	1.027	1.697	-0.5245
Liver, gallbladder and pancreas	-0.216	< 0.001	-0.543	1.386	1.726	-0.5704
Colorectal	-0.808	< 0.001	-0.840	0.825	1.785	-0.8590

[1]Non-linear regression based on the "drug dose to inhibitory response" model, and human development index $(HDI)_{50}$ and slope were the two parameters used. $P < 0.01$ was defined as significantly non-zero β; ANOVA.

Table 2 Actual and predicted mortality-to-incidence ratio values of gastrointestinal cancers in California

Cancer	Incidence (ASR, per 100000)	Mortality (ASR, per 100000)	Actual MIR	Predicted MIR (95%PI)[1]	Δ_{MIR}
All gastrointestinal	74.3	39.6	0.533	0.560 ± 0.118	-0.027
Esophageal	3.8	3.4	0.895	0.803 ± 0.148	0.091
Stomach	7.4	4.3	0.581	0.653 ± 0.158	-0.072
Pancreatic	11.3	10.3	0.912	0.918 ± 0.104	-0.006
Liver	8.4	6.8	0.810	0.807 ± 0.101	0.002
Colorectal	43.4	14.8	0.341	0.416 ± 0.173	-0.075

[1]MIR values were predicted using California's human development index (HDI) (0.907) and the best-fit regression models. Δ_{MIR} = actual MIR-predicted MIR. ASR: Age-standardized rate; MIR: Age-standardized mortality-to-incidence ratio; PI: Prediction interval.

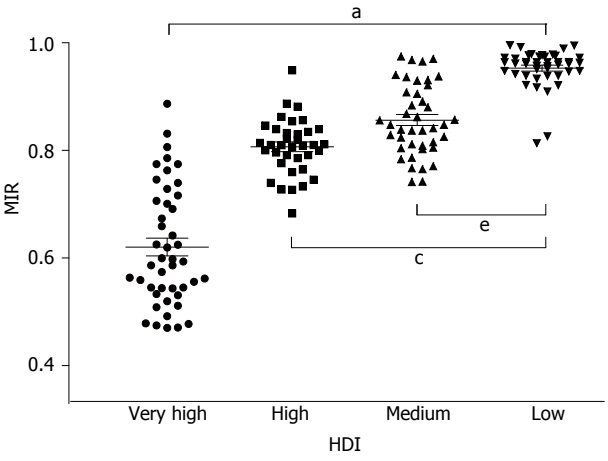


Figure 4 Overall mortality-to-incidence ratio of gastrointestinal cancers in four human development index groups. The mortality-to-incidence ratio (MIR) differs significantly among areas having very high, high, medium and low human development index (HDI); [a]$P < 0.05$ *vs* very high HDI areas; [c]$P < 0.05$ *vs* high HDI areas; and [e]$P < 0.05$ *vs* medium HDI areas; one-way ANOVA followed by the Tukey-Kramer *post-hoc* test.

man development on the effectiveness of healthcare for gastrointestinal cancers, as reflected by the relationship between HDI and MIR, was assumed to bear a similarity to the dose-dependent inhibitory response by anticancer drugs. HDI-to-MIR and dose-to-response patterns both have several characteristics in common, such as (1) MIR or response approaches 1 as HDI or dose approaches 0; (2) MIR or response decreases as HDI or dose increases; and (3) MIR or response approaches 0 as HDI or dose approaches infinity. Non-linear regression using the modified "drug dose to inhibition response" model confirmed the assumption and provided the HDI_{50} value, which was found to be a potential estimate of healthcare effectiveness on gastrointestinal cancers. The progress in screening, diagnostic and therapeutic techniques for colorectal cancer in recent decades[1,29] has resulted in an HDI_{50} of 0.825, which is the lowest value among all the gastrointestinal cancers investigated in this study.

Inequality in healthcare has been regarded as a major cause of variation in the effectiveness of cancer care[30], reflected by the inverse correlation between MIR and HDI. Although eliminating such disparities in healthcare has become the focus of an initiative of healthcare reform in many countries, quality improvement in medical care is not yet obvious[9]. Region- or country-specific disparities in cancer care still exist, even in highly developed countries[7]. Apart from healthcare inequality, the inverse correlation between HDI and MIR is also influenced by the factors such as socioeconomic conditions, lifestyle (particularly diet and tobacco use), and genetic variances among individuals or races[7,9]. Infection with *Helicobacter pylori*, hepatitis virus or other cancer-inducing micro-organisms is another risk factor for gastrointestinal cancers[31-33]. A very recent study analyzed world cancer burden by HDI groups and suggested disparities in cancer distributions[3]. We further demonstrated that HDI influenced cancer MIRs on a country level, which resembled the effect of drug dose on inhibitory response. Therefore, relatively high MIRs indicate the premature mortality from cancer in lower HDI areas. Healthcare disparities emphasize the need for efforts in cancer control in low human development settings.

Cancer health disparities occur not only between countries, but also within a single country[7,34,35]. The health outcomes in the United States were related to socioeconomic factors and racial diversity[9,36]. Health inequality between different socioeconomic levels also contributed to the health disparities observed in the United States. Therefore, we supposed that the observed association between HDI and MIR could be applied to United States states. Prediction based on the modified "drug dose to inhibition response" model turned out to be relatively satisfactory.

The methods used to estimate cancer-specific incidence and mortality rates at the national level in the GLOBOCAN database depend on the availability and accuracy of local data sources[3]. Despite the various provisos concerning data quality and methods of estimation, the estimates in GLOBOCAN are the most accurate that can be made at present, and may be used in the setting of priorities for cancer control actions in different regions and countries of the world[14]. Countries

without high quality data are usually those countries with lower development levels. Limiting analysis to high quality data could eliminate biases due to data inaccuracy, but would lead to excessive absence of epidemiological data in the less developed countries. Since our study aimed to show the disparities of cancer MIRs between low and high HDI countries, the data with relatively low quality were essential to this study and therefore remained in our analysis.

In conclusion, the results of this study obtained by collating excellent data resources revealed an inverse correlation between HDI and MIR at the regional and national levels. This association illustrated that more developed areas tend to have relatively more effective healthcare systems, resulting in low MIRs. Further prediction of the state-specific MIR of gastrointestinal cancers obtained using a fitted non-linear regression model revealed the potential application of HDI for estimation of the MIR.

COMMENTS

Background

Gastrointestinal cancer is a common, highly fatal disease. The expected cancer burden will be a serious public health problem in the coming decade, particularly in developing countries. However, little is known about healthcare disparities in individuals suffering from gastrointestinal cancers.

Research frontiers

There is little knowledge about the healthcare disparities in individuals suffering from gastrointestinal cancers. Inequality in healthcare has been regarded as a major cause of variation in the effectiveness of cancer care. Region- or country-specific disparities in cancer care still exist, even in highly developed countries.

Innovations and breakthroughs

According to the authors of this study, this study is the first to explore the exact relationship between the epidemiology of gastrointestinal cancers and area-specific development disparities. The authors showed the association between the mortality-to-incidence ratios (MIRs) and the human development index (HDI) at the regional and national levels using a modified "drug dose to inhibition response" model. Further prediction of the MIRs for individual United States states on the basis of best-fitted non-linear models showed little deviation from the actual MIRs in the United States.

Applications

Based on the modified "drug dose to inhibition response" model, more developed areas have relatively more effective healthcare systems, resulting in low MIRs. Prediction of the state-specific MIR of gastrointestinal cancers obtained using a fitted non-linear regression model revealed the potential application of HDI for estimation of the MIR.

Terminology

MIR is derived as a surrogate indicator of the effectiveness of the health system, and is proposed as an indirect measure of true biological differences in disease phenotypes or health system-related attributes such as screening, diagnostic modalities, treatment and follow-up. HDI is a composite measure of human development based on the following parameters: a long and healthy life, access to knowledge, and a decent standard of living.

Peer review

The authors, using the GLOBOCAN 2008 database, obtained age-standardized incidence and mortality rates for gastrointestinal cancers. They estimated the MIRs at the regional and national levels, and explored the association between the MIR and development levels as measured by the HDI. Furthermore, they have predicted state-specific MIRs in the United States from the estimated HDI using the fitted non-linear model. Finally, the authors have managed to show an inverse correlation between HDI and MIR at the regional and national levels and that more developed areas tend to have relatively more effective healthcare systems, resulting in low MIRs. Overall, the manuscript is very well written and well organized. The language is satisfactory and the tables along with the figures are well structured.

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P- Reviewers Akbulut S, Koukourakis GV, Motoo Y
S- Editor Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5271

World J Gastroenterol 2013 August 28; 19(32): 5271-5277
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Sessile serrated adenomas in the proximal colon are likely to be flat, large and occur in smokers

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Received: March 19, 2013 Revised: May 15, 2013
Accepted: June 8, 2013
Published online: August 28, 2013

Abstract

AIM: To examine the epidemiology and the morphology of the proximal sessile serrated adenomas (SSAs).

METHODS: We conducted a retrospective study to identify patients with SSAs using a university-based hospital pathology database query from January 2007 to April 2011. Data collected included: age, gender, ethnicity, body mass index, diabetes, smoking, family history of colorectal cancer, aspirin, and statin use. We collected data on morphology of SSAs including site (proximal or distal), size, and endoscopic appearance (flat or protuberant). We also compared proximal SSAs to proximal tubular adenomas detected during same time period.

RESULTS: One hundred and twenty patients with SSAs were identified: 61% were distal and 39% were proximal SSAs. Proximal SSAs were more likely to be flat than distal (100% *vs* 78% respectively; $P = 0.0001$). Proximal SSAs were more likely to occur in smokers (OR = 2.63; 95%CI: 1.17-5.90; $P = 0.02$) and in patients with family history of colorectal cancer (OR = 4.72; 95%CI: 1.43-15.55; $P = 0.01$) compared to distal. Proximal SSAs were statistically more likely to be $\geqslant$ 6 mm in size (OR = 2.94; $P = 0.008$), and also more likely to be large ($\geqslant$ 1 cm) (OR = 4.55; $P = 0.0005$) compared to the distal lesions. Smokers were more likely to have proximal ($P = 0.02$), flat ($P = 0.01$) and large ($P = 0.007$) SSAs compared to non-smokers. Compared to proximal tubular adenomas, proximal SSAs were more likely to be large and occur in smokers.

CONCLUSION: Proximal SSAs which accounted for two-fifths of all SSAs were more likely to present as flat lesions, larger SSAs, and were more likely to occur in smokers and in patients with family history of colorectal cancer. Our data has implications for colorectal cancer screening.

Key words: Proximal; Sessile; Serrated; Adenoma; Colonoscopy; Colorectal cancer; Smoking

Core tip: Sessile serrated adenomas (SSAs) have been implicated in the alternative pathway for colorectal carcinoma. Proximal SSAs might account for higher incidence of interval colorectal cancers (CRC) on the right side given the fact that these are often flat and difficult to detect. Our study is first to compare the morphology and epidemiology of proximal SSAs with distal SSAs.

We found proximal SSAs are more likely to present as flat lesions, larger SSAs, and were more likely to occur in smokers and in patients with family history of CRC. These findings have implications for CRC screening.

Rustagi T, Rangasamy P, Myers M, Sanders M, Vaziri H, Wu GY, Birk JW, Protiva P, Anderson JC. Sessile serrated adenomas in the proximal colon are likely to be flat, large and occur in smokers. *World J Gastroenterol* 2013; 19(32): 5271-5277 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5271.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5271

INTRODUCTION

Colorectal cancer is the fourth most common form of cancer and the second most frequent cause of cancer deaths in the United States[1]. The majority of colorectal cancers arise from the adenoma-carcinoma sequence where mutations in the *APC* gene play an early role. However, an alternative pathway exists in which there is an increased frequency of CpG island methylation of gene promoter. These abnormalities are associated with *BRAF* mutations which have been observed in sessile serrated adenomas (SSAs)[2,3] as well as serrated aberrant crypt foci[4]. Large serrated polyps (≥ 1 cm) have been shown to have a strong association with synchronous advanced colorectal neoplasia[5,6]. SSAs are often flat and proximally located. Interval cancers have been shown to be associated with the methylation pathway[7]. In addition to the fact that they may be difficult to detect, SSAs may provide an explanation for the reason why rates of right sided colorectal neoplasia remain high while the left sided lesions have decreased in patients who have had a colonoscopy in the recent past[8,9].

Very few studies have examined the epidemiology of the various types of serrated polyps. A recent study has shown smoking to be strongly associated with SSAs of all sizes, including the clinically important large (≥ 1 cm) lesions[10]. Multivariate logistic regression found that age, smoking and obesity were statistically significant predictive factors for any SSA as compared to controls[10]. Most of the preceding studies have focused on the relatively common left-sided serrated polyps and little is known about the proximal SSAs. Our goal was to examine the epidemiology and the morphology of the proximal SSA in comparison to the distal lesions.

MATERIALS AND METHODS

Patient selection and data collection

The retrospective study was approved by the Institutional Review Board of the University of Connecticut Health Center. We defined cases as patients diagnosed to have SSAs from January 2007 to April 2011, identified by a pathology database query. We identified all lesions diagnosed by our pathologists as SSA. We excluded the traditional serrated adenomas or the subgroup of serrated polyps that not only share serrated crypt architecture with hyperplastic polyps, but also have cytologic dysplasia. SSAs were those serrated polyps with abnormal proliferation and/or abnormal architecture, but without the cytological dysplasia seen in adenomatous polyps. All of the SSAs were confirmed or had a clinical description that alerted the pathologist that the endoscopist was suspecting a SSA. We defined a large SSA as any SSA of size greater than or equal to 1 cm in diameter.

We collected the following data from the patient's charts: age, gender, ethnicity, height, weight, clinically diagnosed type Ⅱ diabetes mellitus, smoking exposure, a family history of colorectal cancer, lipid profile, use of aspirin, calcium, hormone replacement therapy and statin use. From an electronic database at our University Hospital, we were able to use several different primary care and sub specialty notes to collect and confirm the data. Thus, most of our information had at least one source.

With regard to smoking, we calculated the exposure in the form of pack-years (*i.e.*, number of packs smoked per day multiplied by the number of years smoked). We defined a smoker as someone who smoked at least 20 pack-years or more regardless of whether they quit smoking. Family history of colorectal cancer was defined as having at least one first degree relative or two second degree relatives with the disease. Obesity was defined as a body mass index ≥ 30 kg/m^2. We also randomly selected patients with adenomas who had colonoscopies during the same time period as the patients with serrated lesions.

High-definition (1080i signal) wide-angle (170° field of view) Olympus 180-series colonoscopes (Olympus America, Center Valley, PA, United States) were used to perform all of the colonoscopies. All polyps were photo documented next to a snare catheter for *in vivo* measurement and retrieved for histology, and morphology was classified according to the Japanese Research Society for Cancer of Colon and Rectum guidelines[11,12]. We used a standard method to visualize the polyp for morphologic classification. Specifically, the colon was insufflated so that the polyp was visualized and photo documented in this setting. Any lesion that was determined to be Ip, Is, or Ips was considered to be polypoid or protuberant, and those that were Ⅱa, Ⅱb, or Ⅱc were considered to be flat or non-polypoid. Adenoma size was confirmed by the pathology report[13]. One experienced endoscopist (Anderson JC) confirmed the morphology from the photodocumentation for a representative group of adenomas that were randomly selected from our analyzed sample. The colon was divided into proximal and distal by the splenic flexure which was considered proximal. A colonoscopy was considered complete if the following criteria were fulfilled: transillumination of the right lower quadrant, visualization of the ileocecal valve, or appendiceal orifice.

Statistical analysis

Our main outcomes were detection of SSAs, and proximal SSAs. SPSS version 20.0 (Chicago, IL, United States)

Table 1 Comparison of patient characteristics among proximal and distal sessile serrated adenoma group *n* (%)

	Proximal SSA (*n* = 47)	Distal SSA (*n* = 73)	Univariate OR (95%CI)	*P* value	Multivariate OR (95%CI)
Race (CC)	37 (78.7)	54 (74.0)	1.30 (0.54-3.12)	0.66	-
Gender (male)	21 (44.7)	27 (37.0)	1.38 (0.65-2.90)	0.45	-
Age (yr) (≥ median)	28 (59.6)	40 (54.8)	1.22 (0.58-2.56)	0.71	-
Obesity	22 (46.8)	36 (49.3)	0.90 (0.43-1.88)	0.85	-
Family history	11 (23.4)	5 (6.8)	4.16 (1.34-12.89)	0.01	4.72 (1.43-15.55) *P* = 0.01
Diabetes mellitus	14 (29.8)	23 (31.5)	0.92 (0.42-2.05)	1.00	-
Smoking	30 (63.8)	30 (41.0)	2.53 (1.19-5.39)	0.02	2.63 (1.17-5.90) *P* = 0.02
Triglyceride (mean ± SD, mg/dL)	124.9 ± 63.9	129.7 ± 67.9	-4.80 (-29.38-19.78)	0.69	-
Cholesterol (mean ± SD, mg/dL)	179.1 ± 45.8	180.9 ± 43.8	-1.80 (-18.31-14.71)	0.83	-

SSA: Sessile serrated adenoma.

was used for all statistical analysis. Univariate analyses were performed using Fisher's test or χ^2 for dichotomous variables and unpaired *t*-test for non-parametric continuous variables. After univariate analyses, all variables with a *P* value of 0.10 or less were entered into the equation and only those variables with $P < 0.10$ were used in the final multivariate logistic regression equation to estimate Odds ratios and 95% confidence intervals for proximal SSAs. We considered results to be significant if the *P* value was < 0.05.

RESULTS

From January 2007 to April 2011, 120 patients (mean age: 59.72 ± 10 years, males: 40%) with SSAs were identified. This included 90 patients searched through the same pathology database query that were part of the earlier study focused on identifying risk factors associated with any SSAs[10]. Thirty additional patients were added to this database between October 2010 and April 2011. Proximal SSAs constituted two-fifths (47/120) of all SSAs. Fifty-seven (78%) of the distal lesions were flat as compared to the 47 (100%) proximal lesions which were all flat ($P = 0.0001$). Proximal SSAs were more likely to occur in smokers compared to distal (30/47 *vs* 30/73; $P = 0.02$) as shown in Table 1. Similarly, smokers were more likely to have proximal SSAs compared to non-smokers (30/60 *vs* 17/60; $P = 0.02$). Compared to non-smokers, smokers were also more likely to have flat SSAs (57/60 *vs* 47/60; $P = 0.01$). Proximal SSAs were more likely to be found in subjects with a family history of colorectal cancer compared to distal SSAs (11/47 *vs* 5/73; $P = 0.01$) as shown in Table 1.

We also examined the site of the SSA in relation to the adenoma size and morphology. Proximal SSAs were more likely to be ≥ 6 mm in size and also more likely to be large (≥ 1 cm) compared to the distal lesions, as shown in Table 2. Smokers were significantly more like to have large SSAs (23/60 *vs* 9/60; $P = 0.007$; multivariate OR = 3.93; 95%CI: 1.52-10.17) compared to non-smokers.

We compared SSA group to a control group consisting of 122 patients with conventional tubular adenomas identified from the same time period. Proximal tubular adenoma constituted 64% of all tubular adenomas compared to proximal SSA which constituted 39% of all SSAs. Proximal SSAs were significantly more likely to be flat, large (≥ 1 cm), and occur in smokers compared to the proximal tubular adenomas, as shown in Table 3.

DISCUSSION

Our data suggest that proximal SSAs are more likely to occur in smokers and in patients with family history of colorectal cancer. Proximal SSAs are also more likely to present as large lesions, including the significant (≥ 6 mm) adenomas and clinically important large (> 1 cm) adenomas. In addition, we found proximal SSAs to be more likely to present as flat lesions. To our knowledge, this is the first study examining the morphology of SSAs specifically, and their association with smoking with respect to anatomical location.

We found smokers to have proximal, flat and large SSAs. Smoking has been associated with key mutations in cancer-related genes such as *hMLH1*, CPG island methylation phenotype (CIMP) and *BRAF* mutation, with multiple studies establishing a definitive link between smoking and microsatellite instability-high (MSI-H) colorectal cancers[14-19]. Molecular studies have shown serrated polyps including SSAs to be associated with a higher frequency of CIMP and *BRAF* mutations[3,20-22]. Several large studies have reported the association of serrated adenoma- carcinoma pathway *via* the microsatellite instability[23-28]. With respect to the link between smoking and serrated lesions, multiple studies have shown that cigarette smoking has a stronger association with serrated polyps than it does with adenomatous polyps[29-34]. Wallace *et al*[35] identified smoking as one of the major risks for serrated polyps. Current smokers were found more likely to have proximal nondysplastic serrated polyps in a study by Schreiner *et al*[6]. A recent study by Anderson *et al*[10] demonstrated smoking to be a major risk factor for the presence of SSAs. Our current study further links smoking strongly with proximal SSAs compared to distal lesions. Thus, smoking is not only a major risk factor for all SSAs, but is a much stronger predictor of proximal SSAs. Our study demonstrates smoking to be strongly

Table 2 Comparison of adenoma characteristics in the proximal and distal sessile serrated adenoma *n* (%)

	Proximal SSA (*n* = 47)	Distal SSA (*n* = 73)	Univariate OR (95%CI)	*P* value
Flat SSA	47 (100.0)	57 (78.0)	-	0.0001
⩾ 6 mm SSA	31 (66.0)	29 (39.7)	2.94 (1.37-6.31)	0.0080
⩾ 1 cm SSA	21 (44.7)	11 (15.0)	4.55 (1.92-10.77)	0.0005

SSA: Sessile serrated adenoma.

Table 3 Comparison of patient and adenoma characteristics among proximal sessile serrated adenoma and proximal tubular adenoma groups *n* (%)

	Proximal SSA (*n* = 47)	Proximal TA (*n* = 78)	Univariate OR (95%CI)	*P* value
Family history	11 (23.4)	14 (18.0)	1.40 (0.57-3.40)	0.4900
Smoking	30 (63.8)	26 (33.3)	3.53 (1.65-7.54)	0.0010
Adenoma size ⩾ 1 cm	21 (44.7)	11 (14.1)	4.92 (2.08-11.61)	0.0002
Flat morphology	47 (100.0)	46 (59.0)	-	< 0.00001

SSA: Sessile serrated adenoma; TA: Tubular adenoma.

linked with flat and proximal SSAs, which were more likely to present as large lesions having higher neoplastic potential.

Several studies have explored the association between smoking and anatomical site-specific lesions. Colorectal cancers arising from the serrated pathway that are *BRAF*-mutated, CIMP-high and MSI-H, and are specifically associated with smoking[17,18] occur most often in the proximal colon[36,37]. Limsui *et al*[16] also reported an association between proximal colon cancer and cigarette smoking in a large cohort study of over 37000 women. However, few studies, including a meta-analysis of the association between colorectal cancer and smoking, suggest a specific association with distal/rectal neoplasia[15,38,39]. A recent case-control study by Burnett-Hartman *et al*[29] also reported a stronger association between distal/rectal colorectal polyps and smoking. Botteri *et al*[40] showed a strong association between smoking and cancers in the rectum and proximal colon. They postulated that this could be due to the differential anatomical location of serrated colorectal cancers. Although non-serrated polyps tend to have no site predilection[40-42], studies have reported that serrated neoplasia arise more frequently in the proximal colon and in the rectum[43-45]. Microsatellite instability has been associated with proximal lesions[46,47] and has been shown to develop late in serrated adenoma-carcinoma pathway[3]. This could possibly explain our observation of large and proximal SSAs in smokers. As with microsatellite instability, studies have shown that tumors involving *BRAF* mutations arise more frequently in the proximal colon than in the distal colon[7,48-50]. Our study shows proximal SSAs comprise two-fifths of all SSAs, but are clinically more important given the finding that they are larger and all have flat morphology compared to the distal lesions which were more common. Smoking was found to be a much stronger risk factor for proximal SSAs compared to proximal tubular adenomas, likely due to high frequency of CIMP and *BRAF* mutations which are involved in serrated lesions.

Another interesting observation was the link between family history of colorectal cancer and proximal SSAs on both univariate and multivariate analyses. Family history of colorectal cancer has been shown to be a predictor of proximal significant adenomas on previous studies[51]. Schreiner *et al*[6] also found patients with family history of colorectal cancer to be more likely to have proximal non-dysplastic serrated polyps. However, this study did not include an analysis that distinguished hyperplastic polyps and SSAs. Our study is the first to show similar association of family history of colorectal cancer with proximal SSAs. Anderson and colleagues did not find family history of colorectal cancer to be a risk factor for SSAs compared to controls[10]. This might be because of the relatively small sample size and the fact that distal lesions accounted for two-third of all SSAs. Our results show family history of colorectal cancer is associated with proximal and not distal SSAs. Patients with family history of colorectal cancer might have an alternative involvement of *BRAF*-serrated pathways predisposing them to proximal SSA, which might account for the increased risk of adenoma and colorectal neoplasia.

There are many implications for our findings with respect to colorectal screening. The majority of our SSAs were flat. Those located proximally were all flat as opposed to the distal lesions. These lesions may be difficult to detect and may be associated with synchronous advanced neoplasia[5,6]. Proximal SSAs would be theoretically much more difficult to detect given their location: incomplete colonoscopies, variation in cecal intubation rates, variation in detection of proximal serrated polyps[52]. Given the potential for malignancy of SSAs as well as their proclivity to a flat morphology, these lesions may explain the lack of protection of colonoscopy in the proximal colon. Studies have shown the limitations of colonoscopy in reduction of right sided colon cancers[8,9]. Interval colorectal cancers are three times as likely to occur in the right colon[53] and proximal SSAs might account for significant proportion of these interval colorectal cancers. Recent study by Arain *et al*[7] also found interval cancers to be more likely to arise in the proximal colon and found both CIMP and MSI to be independently associated with interval cancers. This might pose an important concern from a screening perspective. Proximal SSAs are more likely to occur in smokers which may require special screening techniques to identify these lesions in this high risk group. We further divided our SSAs into the larger lesions due to their malignant potential and those > 6 mm. We chose the latter measurement since lesions of this size are considered important clinically with regard to optical colonoscopy as well as computer tomographic colonography (CTC)[54,55]. We observed that most of these lesions were found proximal to the splenic flex-

ure. Therefore, if chromoendoscopy is found to be beneficial in detecting flat adenomas, the entire colon, with special attention to the right side, would be important in smokers and in patients with family history of colorectal cancer. Therefore, great attention to the proximal colon with a detailed evaluation for flat adenomas should be undertaken. Perhaps different techniques, such as special high-definition colonoscopes, narrow band imaging or chromoendoscopy may be required to detect these flat adenomas[56]. The role of CTC in screening smokers for colorectal cancer may also change as it may be more difficult to identify lesions with a flat morphology by this method of screening.

We acknowledge that the retrospective design of the study is a potential limitation for our results. Our retrospective data collection included data regarding known colorectal neoplasia risk factors such as smoking history, family history of colorectal cancer and obesity in addition to medication use, dietary intake, lipid profile and patient demographics. However, we acknowledge that there may have been factors that were missed. Another limitation of this study is the relatively small sample size and single center study.

In conclusion, our study is the first to suggest that proximal SSAs are more likely to present as flat and large adenomas, and also more likely to occur in smokers and in patients with family history of colorectal cancer compared to distal SSAs. Smokers are more likely to have proximal, flat, and large SSAs. Increased malignant potential from larger size and difficulty in detection given their flat morphology might contribute to higher risk of interval colorectal cancer in the proximal colon, particularly in smokers.

COMMENTS

Background

Sessile serrated adenomas (SSAs) have been implicated in the alternative pathway for colorectal carcinoma (CRC) and might account for significant proportion of interval CRC given the fact that these are often flat and difficult to detect. Lesions in this pathway and interval cancers share a common proximal location as well as molecular mutations. Many of the epidemiological studies have focused on the relatively common left-sided serrated polyps and little is known about proximal SSAs.

Research frontiers

Smoking, age, obesity, diabetes have been identified as risk factors for SSAs. Proximal serrated polyps have attracted more attention based on their premalignant potential and their association with synchronous and metachronous lesions.

Innovations and breakthroughs

Their results show differences in risk factors, epidemiology and morphology between proximal and distal SSAs. These novel data show that proximal SSAs are all flat and more likely to present as larger lesions. Proximal SSAs are more likely to occur in smokers and in patients with family history of CRC.

Applications

Smokers are more likely to have proximal SSAs which are flat and larger. This might have implications for CRC screening, recommending use of new or different techniques such as chromoendoscopy in smokers for detection of these lesions which account for significant proportion of interval cancers in the right colon. Future studies should focus on techniques and procedure-related factors to enhance the detection of these clinically important proximal SSAs.

Terminology

Sessile serrated adenoma are characterized by the presence of a disorganized and distorted crypt growth pattern that is usually easily identifiable upon low-power microscopic examination. Crypts, particularly at the basal portion of the polyp, may appear dilated and/or branched, particularly in the horizontal plane, which leads to the formation of "boot", "L", or "anchor"-shaped crypts. The terms "SSAs" and "sessile serrated polyp" are considered synonyms, and both are acceptable. Proximal colon location is defined as proximal to the splenic flexure (transverse colon, ascending colon, cecum, ileocecal valve).

Peer review

This is a nice and well written retrospective case-control study showing that SSAs in the proximal colon were more associated with smoking compared to distal SSAs and tubular adenoma in the proximal colon.

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P- Reviewers Cho YS, Singh S **S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5278

World J Gastroenterol 2013 August 28; 19(32): 5278-5285
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Long-term leukocyte natural α-interferon and ribavirin treatment in hepatitis C virus recurrence after liver transplantation

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Received: February 3, 2013 Revised: June 11, 2013
Accepted: July 4, 2013
Published online: August 28, 2013

Abstract

AIM: To evaluate the effect of long-term treatment with leukocyte natural α-interferon (ln-α-IFN) plus ribavirin (RBV).

METHODS: Forty-six patients with hepatitis C virus (HCV) recurrence received 3 MU three times a week of ln-α-IFN plus RBV for 1 mo; then, patients with good tolerability (n = 30) were switched to daily IFN administration, while the remaining were treated with the same schedule. Patients have been treated for 12 mo after viral clearance while non-responders (NR) entered in the long-term treatment group. Liver biopsies were planned at baseline, 1 year after sustained virological response (SVR) and at 36 mo after start of therapy in NR. MedCalc software package was used for statistical analysis.

RESULTS: About 16.7% of genotype 1-4 and 70% of genotype 2-3 patients achieved SVR. Nine patients withdrew therapy because of non-tolerance or non-compliance. A significant improvement in serum biochemistry and histological activity was observed in all SVR patients and long-term treated; 100% of patients with SVR achieved a histological response (fibrosis stabilization or improvement) with a significant reduction in mean staging value (from 2.1 to 1.0; P = 0.0031); histological response was observed in 84% of long-term treated patients compared to 57% of drop-out. Six patients died during the entire study period (follow-up 40.6 ± 7.7 mo); of them, 5 presented with severe HCV recurrence on enrollment. Diabetes (OR = 0.38, 95%CI: 0.08-0.59, P = 0.01), leukopenia (OR = 0.54, 95%CI: 0.03-0.57, P = 0.03) and severe HCV recurrence (OR = 0.51, 95%CI: 0.25-0.69, P = 0.0003) were variables associated to survival. Long-term treatment was well tolerated; no patients developed rejection or autoimmune disease.

CONCLUSION: Long-term treatment improves histology in SVR patients and slows disease progression also in NR, leading to a reduction in liver decompensation, graft failure and liver-related death.

Key words: Hepatitis C virus; Hepatitis C recurrence; Interferon; Ribavirin; Liver transplantation

Core tip: Recurrent hepatitis C virus hepatitis is associated with a significant increase in morbidity and mortality of transplanted patients; biochemical and necro-inflammatory improves in transplanted patients who achieved a virological response after a course of antiviral treatment. Although the relative small sample size of our study, we demonstrated the efficacy of long-term antiviral treatment on disease progression despite the virological response, without significant side effects.

Tamè M, Buonfiglioli F, Del Gaudio M, Lisotti A, Cecinato P, Colecchia A, Azzaroli F, D'Errico A, Arena R, Calvanese C, Quarneti C, Ballardini G, Pinna AD, Mazzella G. Long-term leukocyte natural α-interferon and ribavirin treatment in hepatitis C virus recurrence after liver transplantation. *World J Gastroenterol* 2013; 19(32): 5278-5285 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5278.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5278

INTRODUCTION

Hepatitis C virus (HCV) -related end-stage liver disease is the main indication for liver transplantation (LT) in Western countries[1]. However, graft re-infection is almost universal, leading to accelerated, severe liver disease with a 30% rate of graft cirrhosis after 5 years[2,3]. Antiviral treatment is indicated for all patients with evidence of recurrent HCV hepatitis[4]; patients with signs of severe HCV recurrence, such as fibrosing cholestatic hepatitis (FCH), must be treated because of the aggressive disease course. The combination of interferon (IFN)-α (both standard and pegylated) plus ribavirin (RBV) is the treatment of choice; however, in the transplant setting, antiviral therapy is less effective. Indeed, IFN plus RBV combination therapy leads to a sustained virological response (SVR) rate of 17%-30%[5,6]. PEG-IFN plus RBV treatment has an SVR rate of approximately 30%[7-9], while in immunocompetent patients, the SVR rate ranges from 40%-82% according to the viral genotype[10]. The decreased efficacy of antiviral treatment in post-transplant patients may be explained by the low tolerability and the high rate of dose reduction and therapy discontinuation due to adverse events[5]. As previously reported, PEG-IFN-based treatment appears to be associated with more hematological and autoimmune adverse events than natural IFN-based therapy[11-13].

Previous studies[14,15] have reported that daily IFN administration leads to good virological and histological outcomes with an acceptable tolerability profile; we hypothesized that daily IFN administration could induce an higher, stable serum IFN concentration, similar to PEG-IFN therapy.

To the best of our knowledge, patients with a SVR to antiviral treatment have improved biochemical and necro-inflammatory activity, while the effect of antiviral treatment on disease progression in non-responders is still controversial[16]. Kornberg *et al*[17] first described the effect of long-term IFN and ribavirin treatment in transplant patients; the authors reported that antiviral maintenance treatment could prevent disease progression, leading to improved long-term survival. Walter *et al*[18] in their retrospective analysis, confirmed the previous results and reported that even in non-responders, long-term antiviral treatment significantly slowed the progression of fibrosis.

Our study aimed to evaluate the virological and histological effects of long-term leukocyte natural α IFN (ln-α-IFN) plus RBV treatment in patients with recurrent HCV hepatitis.

MATERIALS AND METHODS

Patients

From January 2003 to January 2008, 46 patients with recurrent HCV after liver transplantation were prospectively enrolled in our study. The diagnosis of recurrent hepatitis C was made using a combination of biochemical [increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of at least 2x the ULN], virological (positive serum HCV-RNA) and histological findings. Patients with evidence of decompensated liver disease, histological evidence of rejection or drug-related injury, HBsAg positivity, HIV positivity, moderate to severe anemia (Hb < 10 g/dL), leukopenia (WBC < 1500/μL), thrombocytopenia (platelet count < 50000), impaired renal function (creatinine clearance < 50 mL/min), significant history of cardiovascular and psychiatric diseases or ongoing alcohol abuse were excluded.

Treatments

After the diagnosis of recurrent hepatitis C, all of the patients received a standard dose of leuckocyte natural α-IFN (Alfaferone, Alfawasserman, Bologna, Italy), 3 MU three times a week (*tiw*) and ribavirin. After one month of treatment, patients with good tolerance received an increased ln-α-IFN dose of 3 MU daily (Group A), while patients with poor tolerance to the antiviral treatment were maintained on *tiw* dosing (Group B). Tolerance to antiviral treatment was evaluated based on hematological side effects and patient compliance.

Patients who achieved undetectable HCV-RNA levels continued treatment for 12 mo after viral clearance. Non-responders and relapsers entered the long-term treatment group and were treated with ln-α-IFN plus RBV. The S. Orsola-Malpighi internal review board performed a case-by case evaluation for the use of off-label, daily IFN treatment and long-term antiviral therapy. The patients gave informed consent.

Standard immunosuppressive treatment was prescribed to all of the patients at the S. Orsola-Malpighi Hospital; 7 patients received a cyclosporine-based regi-

Table 1 Patients' baseline characteristics (mean ± SE)

Characteristic	Patients (n = 46)
Sex (M/F)	30/16
Age (yr)	57.9 ± 1.28
Time from OLT (mo)	26.3 ± 5.1
ALT (IU/L)	152.3 ± 17.8
AST (IU/L)	105.9 ± 11.5
Gamma-GT (IU/L)	188.7 ± 39.3
Alkaline phosphatases (IU/L)	364.7 ± 39.9
Bilirubin (mg/L)	1.7 ± 0.37
Viral load (log_{10})	6.25 ± 0.09
Genotypes 1/4 *vs* 2/3	36/10
F1/F2/F3/F4	13/16/9/8
Fibrosing cholestatic hepatitis	3
Cyclosporine A *vs* tacrolimus	7/39

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; M/F: Male/female.

men (CyA), while 39 received a tacrolimus-based one (FK).

Patients who presented with anemia or neutropenia received the scheduled IFN and RBV doses; then, erythropoietin was prescribed when the hemoglobin level fell below 10 g/dL, while granulocyte-colony stimulating factor was administered when the neutrophil count was < 700 mmc. When the anemia or neutropenia did not improve with growth factors, the IFN or RBV dose was reduced.

Biochemistry and virological assessment

Quantitative and qualitative HCV-RNA (Versant HCV-RNA 3.0 bDNA, and Versant TMA; Bayer Diagnostics) were measured before starting treatment, after 1 mo and every 3 mo for the first year; then, serum HCV-RNA was checked every 6 mo. Routine blood tests (blood cell counts and liver and renal function tests) were performed at baseline and weekly for the first 4 wk and then monthly.

Liver biopsy

A liver biopsy was performed for all of the patients before enrollment. For patients who achieved a virological response, a liver biopsy was repeated 1 year after the end of treatment; the non-responders and relapsers had a repeat liver biopsy after 30 mo of treatment. The histological staging and grading of chronic, recurrent HCV were evaluated according to the Knodell score[19]. The diagnosis and grading of liver allograft rejection were made according to the Banff international consensus[20].

HCV immunohistochemistry

Five-micron-thick sections of liver tissue were obtained and stored at -80 ℃. HCV immunohistochemistry (IHC) was performed as previously described[21-23]. Reaction positivity was graded according to the percentage of positive cells divided by the total number of hepatocytes (at least 200 cells/high magnification field)[21-23].

Definition

A rapid virological response was defined as HCV-RNA decrease of at least 2 log UI/mL or an undetectable level after 1 mo of treatment. A SVR was defined as a persistently undetectable serum HCV-RNA 6 mo after the end of treatment. The presence of fibrosing cholestatic hepatitis (FCH) or F4 fibrosis on enrollment was considered to be severe, recurrent HCV. A histological response was defined as an improvement or stabilization of liver fibrosis.

Statistical analysis

The data are expressed as the mean ± SE. Group comparisons were calculated using the χ^2 test, the Mann-Whitney test, the Wilcoxon test, t tests (both independent sample t-test and paired t-test), and an ANOVA when appropriate. Clinical events (SVR, and death) were analyzed using Kaplan Meier curves. Logistic regression was used to detect variables that were independently related to the clinical events. Statistical analysis was performed using the MedCalc package v.11.5 for Windows.

RESULTS

Patient characteristics

Forty-six patients (30 males, 57.9 ± 1.28 years old) with post-transplant HCV recurrence were prospectively enrolled; the patients' baseline characteristics are described in Table 1. Eleven patients presented with severe liver disease; 3 patients had FHC, and 8 patients had F4 fibrosis. Thirty-five patients presented with chronic HCV hepatitis with F1-3 fibrosis. Thirty-six patients had HCV genotype 1 or 4, while 10 patients had HCV genotype 2 or 3.

Treatment

The delay between LT and the initiation of treatment was 26.3 ± 5.1 mo. All of the patients (n = 46) received *tiw* IFN plus RBV treatment for the first month; then, 30 patients received 3 MU IFN daily plus RBV (Group A), while 16 continued *tiw* IFN + RBV treatment (Group B). The mean ribavirin dose during the treatment period was 8.4 ± 0.7 mg/kg per day; there was no difference in the RBV dose between Groups A and B.

Virological response

Among the entire population, seventeen patients (37%) achieved undetectable HCV-RNA levels during therapy and continued IFN+RBV treatment for 12 mo after viral clearance (mean 20.7 ± 2.5 mo); 4 of them relapsed after discontinuing treatment and were included in the long-term treatment group. Thirteen patients achieved an SVR: 8 of 30 patients (26.7%) in Group A and 5 of 16 (31.2%) in Group B. No difference between the groups was observed (P > 0.05).

The SVR rate was significantly higher for those with HCV genotype 2 or 3 than genotype 1 or 4 (70.0% *vs* 16.7%, respectively P = 0.0007); the overall SVR rate was 28.3%. Nine patients were rapid virological responders; among them, 7 had HCV genotype 2 or 3, and 2 had genotype 1 or 4. The variables from the univariate

Table 2 Variables associated with sustained virological response according to univariate analysis

Variable	*r*	95%CI	*P*-value
BMI > 25 kg/m^2	0.30	0.01455-0.5458	0.0400
Genotypes 2-3 *vs* 1-4	0.37	0.09103-0.5974	0.0100
RVR	0.54	0.2992-0.7194	0.0001
HCV-RNA clearance during treatment	0.82	0.6948-0.8967	0.0001
CyA *vs* FK immunosuppression	0.41	0.09597-0.6519	0.0120

RVR: Rapid virological response; HCV: Hepatitis C virus; CyA: Cyclosporine; FK: Tacrolimus; BMI: Body mass index.

Table 3 Histological response after treatment (mean ± SE)

	Grading			Staging		
	Before	After	*P*-value	Before	After	*P*-value
Sustained virological response (n = 9)	7.2 ± 0.8	2.6 ± 0.6	0.0039	2.1 ± 0.3	1.0 ± 0.1	0.0031
Long-term treated (n = 19)	7.9 ± 0.7	4.7 ± 0.6	0.0001	2.7 ± 0.3	2.5 ± 0.3	0.0001
Drop out (n = 7)	7.4 ± 1.1	6.0 ± 0.8	NS	2.6 ± 0.6	3.0 ± 0.6	NS

NS: Not significant.

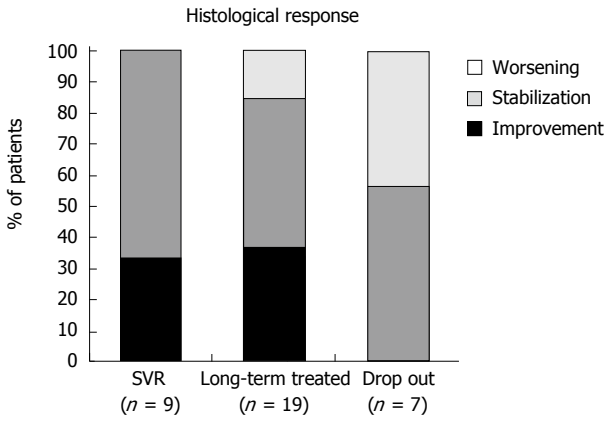

Figure 1 Histological response. Histological response of patients with sustained virological response, long-term treated and drop-out. SVR: Sustained virological response.

analysis associated with an SVR are shown in Table 2; in the multivariate analysis, a rapid virological response (OR = 99.6, 95%CI: 3.1-3190.0, P = 0.0093), a cyclosporine-based immunosuppressive regimen (OR = 685.4, 95%CI: 1.5-314392.9, P = 0.036) and the presence of severe, recurrent HCV (OR = 0.91 95%CI: 0.82-0.99, P = 0.04) were independently associated with a SVR. Eight patients withdrew therapy after 15.2 ± 2.0 mo, one because of moderate-severe anemia and seven because of non-compliance to therapy. Finally, 25 HCV-RNA-positive patients (21 non-responders and 4 relapsers) entered the long-term treatment group and were treated for a mean of 32.4 ± 2.8 mo.

Biochemical response

A significant improvement in ALT was observed in the 13 patients who achieved an SVR (186.1 ± 40.4 IU/L before enrollment *vs* 21.4 ± 2.2 IU/L after treatment, P = 0.0028) and in the 25 long-term treatment patients (154.0 ± 26.6-37.2 ± 4.7 U/L, P = 0.0003). Also, those patients who discontinued therapy (n = 8) showed a biochemical improvement (ALT 169.5 ± 42.7-58.1 ± 3.6 U/L, P = 0.0389). At the end of follow-up, the patients who achieved an SVR had a significantly lower ALT than the other patients (P < 0.05). We also observed that the ALT levels were lower in long-term treatment patients compared to the patients who had to stop treatment (P < 0.05).

Histological response

Among the entire population, 35 patients (9 sustained virological responders, 19 long-term treatment patients and 7 who stopped treatment) underwent a second liver biopsy after 30.3 ± 2.7 mo; seven patients refused the paired biopsy, while four died before the scheduled follow-up. The mean grade and stage are shown in Table 3. The post-treatment grade was significantly lower for the patients who achieved a SVR and received long-term treatment (P = 0.0039 and 0.0001, respectively), while the grade was unchanged for the patients who discontinued therapy.

Liver fibrosis improved (at least 1 stage) in ten of the 35 (28.6%) patients, remained stable in 19 patients (54.3%) and worsened in six patients (17.1%). The histological response in the three groups (responders, long-term treatment and discontinued treatment) is shown in Figures 1 and 2. Liver fibrosis remained stable or improved (histological response) in all of the patients who achieved a SVR (9 of 9, 100%); in this group, the mean post-treatment fibrosis appeared to be significantly lower (P = 0.0031). Interestingly, in the non-sustained virological responders, the histological response was higher in long-term treatment patients (16 of 19) than in the patients who stopped treatment (4 of 7) (84% *vs* 57%, P > 0.05). In the long-term treatment patients, the mean post-treatment fibrosis values were unchanged (2.7 ± 0.3 *vs* 2.5 ± 0.3).

Liver immunohistochemistry

HCV IHC (Figure 3) was performed for all of the liver biopsy (46/46) and paired liver biopsy samples (35/35). The median number of immunoreactive hepatocytes before treatment was 50% (95%CI: 38.9-60.0), and there was no significant difference among the responders, long-term treatment patients and patients who stopped treatment (median 60.0%, from 35.3% to 70.0%; median 45.0%, from 17.6% to 68.3%; median 40.0%, from 4.4% to 80.0%, respectively; P > 0.05).

After treatment, all of the patients who achieved a SVR (9 of 9) had no (0%) immunoreactive hepatocytes in the liver samples (P = 0.0002). Interestingly, the non-responders who received long-term treatment had a significant reduction (P = 0.001) in immunoreactive hepatocytes (before treatment: median 45%, from 17.6% to 68.3%; after treatment: median 0.0%, from 0.0% to

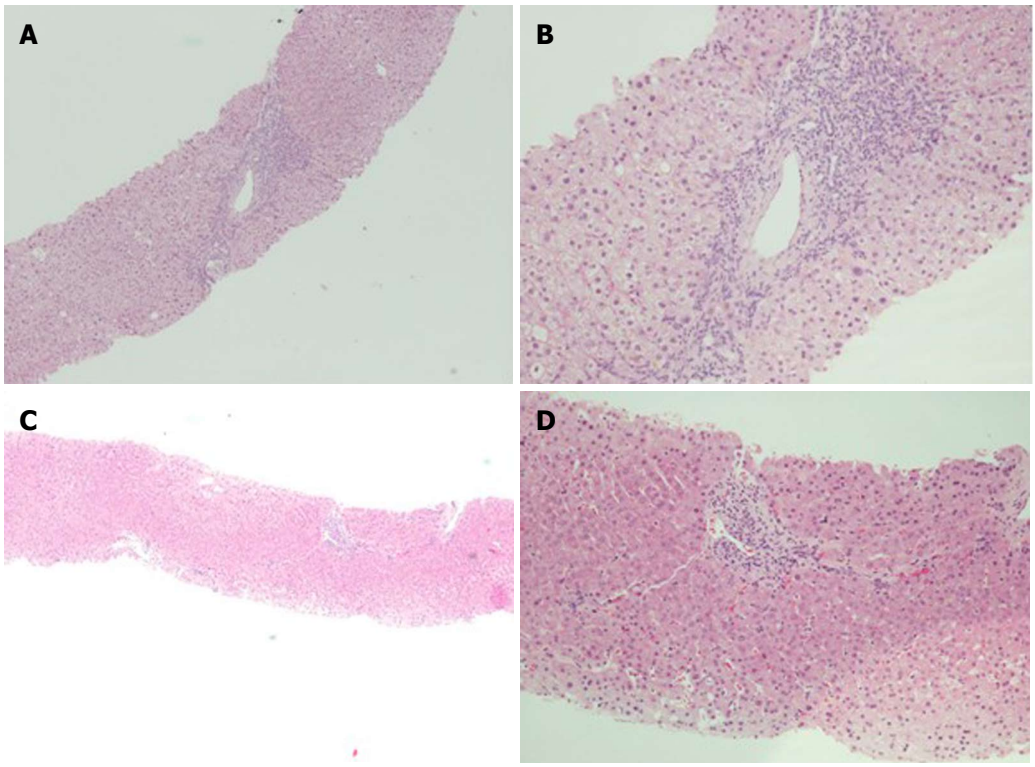

Figure 2 Liver histology. Liver histology before and after treatment in sustained virological response and Long-term treated patients: liver histology from a patient before treatment (A, B) and liver histology of the same patient (non responder) after long-term treatment (C, D). A, C: Hematoxylin and eosin (HE), × 10; B, D: HE, × 20.

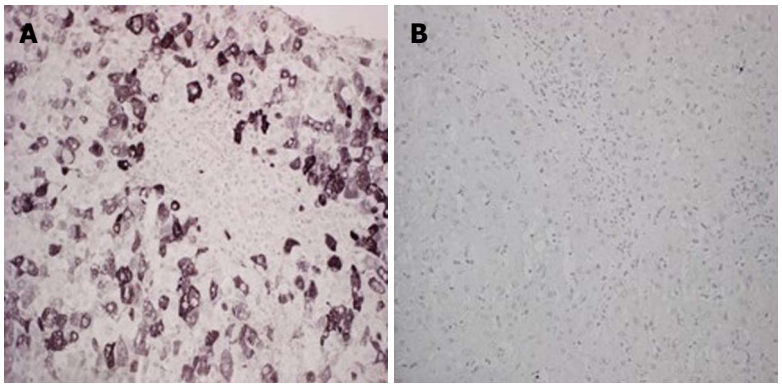

Figure 3 Hepatitis C virus immunohistochemistry. Lobular areas from serial biopsies of the same patient, showing the cytoplasmic positivity of hepatocyte for hepatitis C virus antigens before (A) and after treatment (B). Immunohistochemistry, × 20.

14.1%). No significant difference was observed before and after treatment in the patients who stopped treatment (before: median 40.0%, from 4.4% to 80.0%; after: median 20.0%, from 10.0% to 33.1%; $P > 0.05$).

Tolerability

Treatment was generally well tolerated; 29 (63%) patients, during combination therapy, required growth factors with 28 (61%) patients receiving erythropoietin for anemia and thirteen (28%) receiving G-CSF for neutropenia. Despite the use of grow factors, one patient withdrew from treatment due to moderate-severe anemia (no need for blood transfusion or hospitalization). No patient developed autoimmune disease or graft rejection.

Survival

Six patients died during the study period (follow-up 40.6 ± 7.7 mo). Two patients with a severe HCV recurrence (FCH), one who was a non-responder and another who stopped therapy, died because of a severe infection (encephalitis and cholangitis). Two patients (basal fibrosis F4), who were non-responders (1 long-term treatment and 1 who stopped treatment), died from liver decompensation. One patient with FCH on enrollment received long-term treatment for 40 mo and died 44 mo after enrollment due to liver decompensation. One patient with a mild HCV recurrence died due to a myocardial infarction.

The presence of diabetes (OR = 0.38, 95%CI: 0.08-0.59;

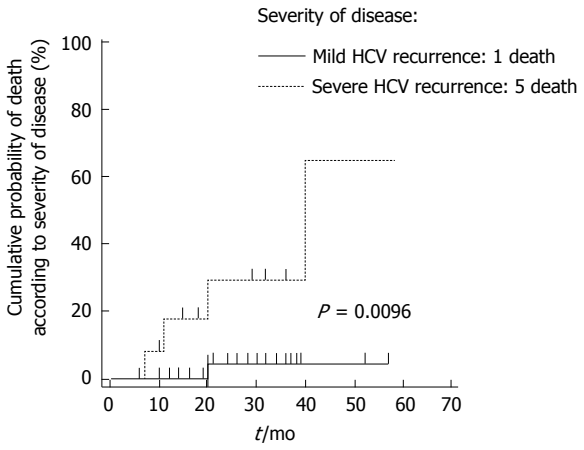

Figure 4 Survival analysis (Kaplan-Maier curve) according to presence of severe hepatitis C virus recurrence. HCV: Hepatitis C virus.

$P = 0.01$), leukopenia (OR = 0.54, 95%CI: 0.03-0.57; $P = 0.03$) or severe HCV recurrence (OR = 0.51, 95%CI: 0.25-0.69; $P = 0.0003$) were associated with survival; in a multivariate analysis, the presence of a severe HCV recurrence (OR = 29.6, 95%CI: 2.4-371.2; $P = 0.0086$) was the only variable to be independently associated with death. Kaplan-Maier analysis (curve shown in Figure 4) demonstrated an increased risk of death ($P = 0.0096$) for patients with a severe, recurrent HCV compared to patients with a mild recurrence. No difference between the survival of patients undergoing long-term treatment and those who discontinued treatment was observed.

DISCUSSION

Recurrent HCV hepatitis is associated with a significant increase in the morbidity and mortality of transplanted patients due to the early development of graft cirrhosis. In the post-transplant setting, the goals of antiviral treatment are to induce viral eradication and to slow disease progression.

Previous studies[5,24-27] have reported biochemical and necro-inflammatory improvement in transplanted patients who achieved a virological response after a course of antiviral treatment; however, the response rate is still unsatisfactory (17%-30% with IFN plus RBV; 18%-45% with PEG-IFN plus RBV)[5-9]. Among the non-responders, a significant number of patients will develop of graft cirrhosis and liver-related death in a few years[2,3]. To slow disease progression, the efficacy of antiviral maintenance therapy was evaluated in two studies[17,18], which showed preliminary evidence of benefit. Our study aimed to evaluate the efficacy of long-term treatment with ln-α-IFN plus ribavirin.

We reported an overall SVR rate of 28.3%; our response rate was similar to those observed with IFN-based and PEG-IFN-based regimens[5-9]. We did not observe any different virological outcomes between patients receiving daily and *tiw* IFN; this result could be due to variation in the HCV genotype distribution. Six patients of 16 (37.5%) in the Group B had a favorable genotype (2 or 3).

The role of cyclosporine in patients with HCV recurrence is still controversial. Our previous experience has demonstrated that the type of immunosuppression during antiviral treatment may predict the SVR[28], but a meta-analysis failed to demonstrate a significant difference in clinical outcome (graft survival and mortality)[29]. Our results showed that a cyclosporine-based immunosuppressive regimen correlated with an increased SVR rate, although there was a small number of CyA-treated patients.

The potential efficacy of pegylated IFN-based antiviral treatments in the transplant setting is limited by poor tolerability and a high rate of adverse events (hematological, autoimmune and rejection) leading to dose reduction and/or therapy discontinuation. Moreover, in our center, we previously experienced several (9 of 44 patients) *de novo* cases of autoimmune hepatitis during PEG-IFN plus ribavirin treatment[12]; therefore, in this study, to reduce adverse events and increase patient tolerability, we used a natural IFN-based regimen. As expected, we observed a good safety and tolerability profile; no patient developed autoimmune disease or graft rejection, while only one (2.2%) stopped treatment due to anemia.

Histological analysis from paired liver biopsy samples showed a reduction in necro-inflammatory activity in the patients who cleared HCV-RNA and those who received a long-term course of therapy. As in the non-transplant setting, achievement of complete viral eradication led to an improvement in liver inflammation, biochemically and histologically. Also, we observed a significant decrease in activity scores (from 7.9 ± 0.7 to 4.7 ± 0.6; $P = 0.0001$) in the non-responders who received long-term treatment with IFN plus RBV.

To evaluate the anti-viral and anti-inflammatory effects of IFN plus RBV treatment, we tested, on paired liver tissue samples, hepatocyte expression of viral proteins using IHC analysis as previously described by Ballardini *et al*[21,22]. As expected, our results showed that patients who cleared HCV did not have HCV-positive hepatocytes in their liver biopsies, while the non-responders who had interrupted antiviral treatment did not have reduced HCV protein expression. Interestingly, the non-responders who received a long-term course of therapy had a significantly reduced percentage of HCV-positive hepatocytes (median: 45%-0%), leading to a significant reduction in liver inflammation. To exclude sampling error, liver HCV-RNA was quantified in those cases; in all of the liver biopsies, HCV-RNA was detected. We hypothesized that the effect of IFN treatment, even in the non-responders, could reduce liver tissue inflammation by reducing the degree of hepatitis C viral antigen staining[30].

The role of antiviral treatment on disease progression is still debated. Patients who achieve an SVR have been shown to have delayed fibrosis progression[24], while some authors have also observed fibrosis regression[16]. A pivotal role for antiviral therapy was demonstrated by a previous study that reported treatment was the only variable to be independently associated with histological

improvement or stabilization among patients with HCV recurrence[16].

In our experience, nine patients who cleared HCV had a significantly reduced staging score on liver biopsy; moreover, a significant percentage (84%) of long-term treatment patients had a histological response despite the lack of viral clearance. Although there was a small number of patients, these results suggest the efficacy of long-term antiviral treatment on disease progression independent of a virological response.

The presence of severe HCV recurrence (histological cirrhosis, cholestatic hepatitis or FCH) is associated with a worse clinical outcome; as in the non-transplant setting[31,32], patients with advanced disease have a reduced SVR rate, due to increased averse events and therapy discontinuation. Moreover, we found that severe HCV recurrence is the only variable to be independently related to the risk of death. These findings suggest that recurrent HCV hepatitis should be treated at the onset of biochemical and histological signs to improve virological and clinical outcomes.

In conclusion, long-term treatment with ln-α-IFN plus ribavirin was able to improve histological staging in SVR patients, slow disease progression in non-responders, and demonstrate a good safety and tolerability profile. These findings suggest the importance of long-term treatment for HCV recurrence; this treatment seems to be able to reduce liver decompensation, graft failure and liver-related death.

ACKNOWLEDGMENTS

We thank Bologna Liver Transplant Group: Pietro Andreone, Giampaolo Bianchi, Sonia Berardi, Maurizio Biselli, Stefano Brillanti, Matteo Cescon, Alessandro Cucchetti, Giorgio Ercolani, Gian Luca Grazi, Marco Lenzi, Simona Leoni, Francesca Lodato, Maria Crisitina Morelli, Fabio Piscaglia, Matteo Ravaioli, Claudia Sama, Gabriella Verucchi, Marco Vivarelli.

COMMENTS

Background

Hepatitis C virus (HCV) graft re-infection after liver transplantation is almost universal, leading to accelerated, severe liver disease; moreover, antiviral therapy in this setting is less effective. Only patients who achieve a virological response have an improvement in biochemical and necro-inflammatory activity, while the effect of antiviral treatment on disease progression in non-responders is still controversial.

Research frontiers

The use of long-term maintenance therapy in transplanted patients who do not achieve a virological response is still debated. Some authors have reported that antiviral maintenance treatment could prevent disease progression, leading to an improvement in long-term survival.

Innovations and breakthroughs

The results support the concept that long-term antiviral treatment leads to a better histological outcome even in patients who do not achieve viral clearance; therefore, long-term antiviral treatment improves disease progression, leading to a better clinical outcome.

Applications

New direct antiviral agents are changing the approach to HCV treatment, including in transplanted patients; however, the management of patients who do not achieve a viral response will be a future clinical challenge. The results supported the safety and tolerability of long-term treatment with interferon and ribavirin in patients who did not respond to therapy. We demonstrated that, even in non-responders, long-term treatment improves clinical and histological outcomes.

Peer review

As a retrospective clinical study on patients with HCV recurrence after liver transplantation, the results proved the efficacy of long-term antiviral treatment with leukocyte natural α-interferon and ribavirin on disease progression and showed a good safety and tolerability profile.

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P- Reviewers Lu XM, Yu B
S- Editor Zhai HH **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5286

World J Gastroenterol 2013 August 28; 19(32): 5286-5294
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Somatic molecular changes and histo-pathological features of colorectal cancer in Tunisia

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Supported by In part by a grant from the Tunisian Government (to Aissi S)
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Received: March 18, 2013 Revised: May 14, 2013
Accepted: July 4, 2013
Published online: August 28, 2013

Abstract

AIM: To determine correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC).

METHODS: Histo-pathological features and molecular changes [*KRAS*, *BRAF* and *CTNNB1* genes mutations, microsatellite instability (MSI) phenotype, expression of mismatch repair (MMR) and mucin (MUC) 5AC proteins, mutation and expression analysis of *TP53*, *MLH1* promoter hypermethylation analysis] were examined in a series of 51 unselected Tunisian CRC patients, 10 of them had a proven or probable hereditary disease, on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

RESULTS: As expected, MSI and MMR expression loss were associated to the presence of familial CRC (75% *vs* 9%, $P < 0.001$). However, no significant associations have been detected between personal or familial cancer history and *KRAS* (codons 12 and 13) or *TP53* (exons 4-9) alterations. A significant inverse relationship has been observed between the presence of MSI and TP53 accumulation (10.0% *vs* 48.8%, $P = 0.0335$) in CRC tumors, suggesting different molecular pathways to CRC that in turn may reflect different environmental exposures. Interestingly, MUC5AC expression was significantly associated to the presence of MSI (46.7% *vs* 8.3%, $P = 0.0039$), MMR expression loss (46.7% *vs* 8.3%, $P = 0.0039$) and the presence of familial CRC (63% *vs* 23%, $P = 0.039$).

CONCLUSION: These findings suggest that MUC5AC expression analysis may be useful in the screening of Tunisian patients with high risk of CRC.

Key words: DNA mismatch repair; *KRAS*; *TP53*; Mucin 5AC

Core tip: This study reports, for the first time in Tunisia, the value of various histo-pathologic features and

somatic molecular changes [*BRAF*, *KRAS*, *CTNNB1*, *TP53*, mismatch repair (MMR) expression, microsatellite instability (MSI), MLH1 promoter methylation] in distinguishing patients with hereditary non polyposis colorectal cancer. Our results revealed that MUC5AC expression was significantly associated with the presence of MSI (46.7% *vs* 8.3%, P = 0.0039), MMR expression loss (46.7% *vs* 8.3%, P = 0.0039) and the presence of familial colorectal cancer (63% *vs* 23%, P = 0.039). These findings suggest that mucin 5AC expression analysis may be useful in the screening of Tunisian patients with high risk of colorectal cancer.

Aissi S, Buisine MP, Zerimech F, Kourda N, Moussa A, Manai M, Porchet N. Somatic molecular changes and histo-pathological features of colorectal cancer in Tunisia. *World J Gastroenterol* 2013; 19(32): 5286-5294 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5286.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5286

INTRODUCTION

Colorectal cancer (CRC) is a complex biological process involving many genes. Intensive screening for genetic alteration in CRC led to the identification of at least two different molecular mechanisms implicated in CRC carcinogenesis: chromosomal (CIN) and microsatellite instabilities (MSI). The CIN pathway is found in about 80% of sporadic CRC and in familial adenomatous polyposis[1]. It involves chromosomal allelic losses[2,3]. The MSI pathway is found in most cases of hereditary non-polyposis colorectal cancer (HNPCC) and in 12% of sporadic CRC. It involves inactivation of DNA mismatch repair (*MMR*) genes. The presence of MMR deficiency leads to the accumulation of mutations in mononuclear tracts in the coding region of genes controlling cell cycle[4]. Although CRC shows genetic heterogeneity, the same four different signalling pathways could be implicated in tumor progression. The WNT/Wingless pathway could be activated through an *APC* mutation in CIN tumors or through a *CTNNB1* stabilizing mutation in MSI tumors[5]. *CTNNB1* and *APC* mutations were observed as early as the adenomatous stage of CRC neoplasia. The transforming growth factor beta (TGFβ) pathway is driven by *SMAD2* or *SMAD4* inactivating mutation in CIN tumors[6] or by a frame-shift mutation in the TGFβ type Ⅱ receptor in MSI tumors[7]. The RAS-MAP kinase pathway is activated by *KRAS* mutations in CIN[8] or by *BRAF* mutations in sporadic MSI tumors. Alteration of these genes correlated closely with the progression of the adenoma to cancer. The TP53 pathway is inactivated by *TP53* mutations in CIN tumors or by *BAX* inactivating mutation in MSI tumors. These alterations contribute to the adenoma-carcinoma transition. More recently, the existence of a third phenotype was suggested. The main alteration associated with this group of tumors is the hypermethylation of the promoter region of numerous genes, leading to their inactivation[9,10]. Activating somatic mutation of *BRAF* gene has been reported in 15% of sporadic tumors with MSI due to *MLH1* hypermethylation and never in tumors from HNPCC families with *MLH1* and *MSH2* germline mutations[11]. MMR germline mutations detections is an important supplement to HNPCC clinical diagnosis. It enables at-risk and mutation-positive relatives to be informed about their cancer risks and to benefit from intensive surveillance programs that have been proven to reduce the incidence of CRC[12]. However, germline tests are time-consuming and costly due to heterogeneity of mutations. In addition, MMR germline mutations are not always detected in Amsterdam positive families (sensibility, 50%-78%)[13]. The difference in somatic mutation status between sporadic CRC and HNPCC-related cancers may prove helpful in distinguishing HNPCC patients. In this study, we analysed for the first time in Tunisia the value of various histo-pathologic features and somatic mutations of 51 CRC cases in predicting CRC susceptibility.

MATERIALS AND METHODS

Patients and tissue specimens

Fifty-one formalin-fixed, paraffin embedded primary colorectal carcinomas and paired normal bowel of 51 different patients who had undergone colonic resection for the treatment of CRC were retrieved by retrospective review of the pathology archives. Ten of these patients were previously characterized for MMR germline mutations associated to Lynch syndrome[14]. Patients were evaluated according the revised Bethesda guidelines for the identification of HNPCC patients[15]. MSI testing, immunohistochemistry and somatic mutational analysis were performed in all patients regardless of age, personal or family history of cancer, and tumor characteristics.

DNA preparation

DNA was extracted from paraffin-embedded tissue samples of primary CRC and paired normal bowel using the DNeasy® tissue kit (Qiagen, Courtaboeuf, France).

MSI analysis

MSI was assessed using a set of five mononucleotide markers (BAT25, BAT26, NR21, NR22, NR24)[15,16].

Expression of MMR proteins

MMR was assessed by immunohistochemistry as previously described[16]. Immunohistochemistry for mucin (MUC) 5AC and TP53. Tumor sections were analysed using mouse monoclonal antibody against p53 (clone DO-7, Dakocytomation) and MUC5AC (clone CLH2, Novocastra). For TP53, a tumor was scored as TP53 overexpression-positive if nuclear staining was seen in more than 20% of the neoplastic cells in the absence of staining in the tumor adjacent cells. For MUC5AC, which is never expressed in normal colon mucosa[17], expression was interpreted as positive if more than 10% of tumor

Table 1 Clinical and histo-pathological characteristics of the 51 colorectal cancer patients *n* (%)

Characteristic	Patient
Age of onset of the first cancer (range) (yr)	51 (17-85)
⩽ 50	25 (49.0)
> 50	26 (51.0)
Sex	
Male	30 (58.8)
Female	21(41.2)
Site of the first CRC	
Right colon	14 (27.5)
Left colon	16 (31.4)
Rectum	21 (41.2)
TNM tumor stage	
Ⅰ	3 (5.9)
Ⅱ	24 (47.1)
Ⅲ	20 (39.2)
Ⅳ	3 (5.9)
Others	1 (2.0)
Degree of differentiation	
Well	33 (64.7)
Moderate	14 (27.5)
Poor	2 (3.9)
Mucinous CRC	2 (3.9)
Mucinous carcinoma type	
⩾ 50%	14 (27.5)
⩽ 50%	37 (72.5)
Signet ring cell carcinoma	2 (3.9)
Tumor infiltrating lymphocyte	
Crohn's–like reaction	2 (3.9)
Intra epithelial lymphocytes	1 (2.0)
Lymphoïde peritumoral reaction	10 (19.6)
Synchronous CRC	3 (5.9)
Metachronous CRC and HNPCC related cancer	3 (5.9)
Fulfillment of guidelines	
Amsterdam	3 (5.9)
Revised Bethesda	22 (43.1)
B1	22
B2	3
B3	11
B4	1
B5	1

CRC: Colorectal cancer; HNPCC: Hereditary nonpolyposis colorectal cancer; TNM: Tumor node metastasis.

cells displayed cytoplasmic staining in the absence of staining in the tumor adjacent cells.

TP53 mutations screening

Primers were designated for the coding regions and exon-intron boundaries of exons 5 to 8. Exons 4 and 9 were only analysed on those samples negative for mutations in exons 5-8. Primer sequences and polymerase chain reaction (PCR) conditions are available on request.

Mutation screening for KRAS, CTNNB1 and BRAF genes

KRAS (codon 12, 13), *BRAF* (exon 15) and *CTNNB1* (β-catenin) (exon 3) were screened in each CRC cancer using direct sequencing in forward and reverse orientations. Primer sequences and PCR conditions are available on request.

MLH1 promoter methylation assay

Genomic DNA obtained from paraffin-embedded tissue section was modified with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) according to the specifications of the manufacturer. Primer sequences for methylation-specific PCR were modified from Grady *et al*[18].

Statistical analysis

Continuous variables are described as mean and range (min-max) and categorical variables as frequencies and percentages. The association between the different measured parameters was tested using non parametric tests. The difference between two independent groups was determinate by Mann-Whitney *U*-test and the significance of differences between more than two groups was calculated using Kruskal-Wallis test. Categorical data were compared by χ^2 appropriate or Fisher exact tests. A *P*-value < 0.05 was considered as statistically significant. Statistical analyses were performed with SPSS version 15.0 (SPSS, Chicago, IL, United States).

RESULTS

Clinical and pathological features

Demographic, clinical and tumor-related characteristics of the study group are summarised in Table 1. Twenty-five (49.0%) probands were under 50 years of age, including 12 (23.5%) under 40 years of age and 5 (9.8%) under 30 years of age; 26 (51%) aged more than 50 years, including 20 (39.2%) aged more than 60 years. Six patients (11.8%) had a personal history of synchronous/metachronous CRC tumors (4 cases) or previous primary CRC and HNPCC-related extracolonic tumors (2 cases). In 8 cases (16%), the proband was found to have at least one first-degree relative with CRC and/or HNPCC-related extracolonic cancers. In total, 25 (49%) of the 51 CRC patients belonged to families fulfilling the Amsterdam Criteria[19] for the clinical definition of HNPCC or fulfilled at least one criterion of the revised Bethesda criteria for the identification of HNPCC patients[15]. Criterion 1 was the most commonly satisfied Bethesda criterion (22/51, 43.1%). Clinical data analysis revealed that CRC was essentially right sided for patients having at least one first- or second-degree relative with CRC; whereas cancer was more frequently left sided or rectal for patients without a familial history of CRC ($P = 0.039$) (Table 2). However, no significant difference in tumor site was seen when Bethesda criteria where considered. The Bethesda-positives CRC tumors same to be associated to a more advanced stage of the disease ($P = 0.050$) (Table 2). However, no statistical difference has been seen when familial history was considered (Table 2).

Pattern and frequency of MSI

MSI-high (MSI-H) phenotype was detected in 10 (19.6%) of the 51 tested tumors. All the MSI tumors showed instability in all 5 analysed markers. Eight patients (8/25; 32%) were Bethesda-positives and only 2 (2/26; 8%) were Bethesda-negatives (Table 2). For the remaining 41 cases, the tumors were microsatellite stable (MSS) including 1 (1/3, 33%) Amsterdam I-positive patient and 16 (16/25,

Table 2 Statistical analysis of clinicopathological parameters of the 51 colorectal cancer studied tumors as a function of tumoral phenotype *n* (%)

	Mutation MMR -	Mutation MMR +	Family history of colorectal cancer		*P*	Ams - and Beth -	Ams - and Beth +	*P*	Ams (- or +) and Beth +	*P*
	(*n* = 6)	(*n* = 4)	Yes (*n* = 8)	No (*n* = 43)		(*n* = 26)	(*n* = 22)		(*n* = 25)	
Mutation geminale										
MMR+			4	0		0	2		4	
MMR-			3	3		0	5		6	
Site of tumor (CCR)					NS			NS		NS
Right colon	1	3	5 (63)	10 (23)		5 (19)	9 (41)		10 (40)	
Left colon	2	0	1 (13)	14 (33)		8 (31)	7 (32)		7 (28)	
Rectum	3	1	2 (25)	19 (44)		13 (50)	6 (27)		8 (32)	
Left colon + rectum	5	1	3 (38)	33 (77)	0.039	21 (81)	13 (59)	NS	15 (60)	NS
Right + left colon	3	3	6 (75)	24 (56)	NS	13 (50)	16 (73)	NS	17 (68)	NS
TNM Stage					NS			NS		NS
Ⅰ	0	1	1 (14)	2 (5)		2 (8)	0 (0)		1 (4)	
Ⅱ	1	0	2 (29)	23 (53)		16 (62)	9 (41)		9 (38)	
Ⅲ	4	3	4 (57)	15 (35)		7 (27)	11 (50)		12 (50)	
Ⅳ	0	0	0 (0)	3 (7)		1 (4)	2 (9)		2 (8)	
Ⅰ / Ⅱ	1	1	3 (43)	25 (58)	NS	18 (69)	9 (41)		10 (42)	
Ⅲ / Ⅳ	4	3	4 (57)	18 (42)		8 (31)	13 (59)	NS	14 (58)	0.050
Microsatellite instability					< 0.001			NS		0.038
MSI (MSI-L or MSI-H)	1	4	6 (75)	4 (9)		2 (8)	6 (27)		8 (32)	
MSS (MSI-L or MSS)	5	0	2 (25)	39 (91)		24 (92)	16 (73)		17 (68)	
Somatic mutations										
TP53	6	0	3 (38)	25 (64)	NS	14 (61)	13 (62)	NS	14 (58)	NS
KRAS	1	2	2 (25)	14 (33)	NS	9 (35)	5 (23)	NS	7 (28)	
BRAF	0	0	0 (0)	1 (2)		1 (4)	0 (5)		0 (0)	
CTNNB1	0	0	0 (0)	1 (2)		0 (0)	1 (5)		1 (4)	
Immunohistochemistry										
Loss of MMR	1	4	5 (50)	5 (50)	NS	2 (8)	6 (27)	NS	8 (32)	0.038
Overexpression of p53	5	0	2 (25)	19 (44)	NS	9 (35)	11 (50)	NS	12 (48)	NS
Overexpression of MUC5AC	2	3	5 (63)	10 (23)	0.039	6 (23)	8 (36)	NS	9 (36)	NS

Ams: Extented Amsterdam Ⅱ criteria; NS: Not statistically significant; Beth: Revised Bethesda Guidelines; MMR: Mismatch repair; CCR: Colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stable; MUC: Mucin.

64%) Bethesda-positives patients. Six (27%) of the 22 Bethesda-positives Amsterdam-negatives patients showed MSI in tumor tissue. Hence, the sensitivity and the specificity of the Bethesda criteria in the prediction of MSI were 80% and 60%, respectively. MSI was also observed in 3 sporadic CRC cases (3/49, 6%). Other laboratories have demonstrated a frequency of MSI between 10% and 20% amongst sporadic CRC cases. Therefore, our results are comparable with results from other series[20,21]. CRC was diagnosed before 50 years of age in 80% (8/10) of the patients with MSI (Table 3). The mean age at tumor diagnosis in MSS patients was higher than in MSI patients [56.2 years (range 17-85 years) *vs* 42.4 years (range 18-72 years)] (Table 3). Two of the 10 MSI patients (20%) had synchronous/metachronous colorectal cancer and no one had additional extracolonic cancer. Mucinous colloid component was significantly more important in MSI-H tumors ($P = 0.0178$) (Table 3). No significant associations were observed between MSI phenotype and sex, tumor site and tumor node metastasis (TNM) stage (Table 3). However, MSI-H tumors have been reported to be more frequent in the proximal colon[22]. A *KRAS* somatic mutation was detected in 4 (4/10, 40%) MSI-H tumors (Table 4): 3 were located at codon 13 (*p*.Gly13Asp) and 1 was at codon 12 (*p*.Gly12Asp). No significant association has been detected between MSI and *KRAS* alterations (Table 4). However, an inverse correlation came to exist between MSI-H phenotype and TP53 overexpression ($P = 0.0335$) (Table 4). On the other hand, MUC5AC abnormal expression was significantly more frequent in MSI tumors compared to MSS tumors ($P = 0.0039$) (Table 4). This result was in accordance with data reported by Biemer-Hüttmann *et al*[23].

MMR protein expression

Forty-one of the 51 (80.4%) analysed CRCs exhibited normal MMR protein expression. Of the remaining 10 (19.6%) CRCs, 8 (80%) showed a combined MLH1 and PMS2 proteins expression loss suggesting an *MLH1* deleterious mutation, 1 (10%) showed a combined MSH2 and MSH6 proteins expression loss, hardly suggesting an *MSH2* deleterious mutation (or *MSH6*, eventually), while just 1 (10%) demonstrated loss of only MSH6 protein, suggesting an *MSH6* deleterious mutation. Two (2/10, 20%) of these patients were Amsterdam-positives whereas 8 (8/10, 80%) where Amsterdam-negatives (Table 2). Five patients with MMR proteins expression loss had a family history of cancer. Of the 8 cases with MLH1 expression loss, 2 (2/8, 25%) had an Amsterdam-positive family history. For MSH2 and/or MSH6 none of the 2 cases with expression loss had a cancer family history. The 10 tumors with MMR expression loss corresponded

Table 3 Comparison of the somatic phenotype and genotype as a function of the patient's clinical characteristics

	MSI (n = 10)	*KRAS* mutations (n = 16)	*TP53* mutations (n = 28)	TP53 overexpression (n = 21)	MUC5AC overexpression (n = 15)
Mean age at diagnosis (range), yr	45 (18-72)	51.5 (18-85)	48.5 (18-79)	49.5 (24-75)	50 (24-76)
Sex					
Males	80.00%	75.00%	57.10%	52.40%	66.70%
Females	20.00%	25.00%	42.90%	47.60%	33.30%
Tumor site[1]					
Proximal	50.00%	18.80%	21.40%	28.60%	33.30%
Distal	50.00%	81.30%	78.60%	71.40%	66.70%
TNM stage[2]					
I	10.00%	6.30%	0.00%	0.00%	0.00%
II	40.00%	43.80%	48.10%	38.10%	40.00%
III	50.00%	50.00%	44.40%	47.60%	53.30%
IV	0.00%	0.00%	7.40%	9.50%	6.70%

[1]Proximal, right colon; distal, left colon + rectum; [2]Tumor, node, metastasis (TNM) stage was unknown for one patient; MSI: Microsatellite instability; MUC: Mucin.

Table 4 Comparison of the microsatellite instability phenotype as a function of tumoral parameters

	MSI-H tumors (n = 10)	MSS tumors (n = 41)	P
MMR expression	100.00%	0.00%	< 0.0001[1]
KRAS mutations	40.00%	29.30%	NS
TP53 mutations	30.00%	67.60%	NS
TP53 surexpression	10.00%	48.80%	0.0335[1]
MUC5AC surexpression	70.00%	19.50%	0.0039[1]

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair; MSS: Microsatellite stable.

Table 5 Comparison of *TP53* somatic mutations as a function of tumoral parameters

	Presence of *TP53* mutations (n = 28)	Absence of *TP53* mutations (n = 19)	P
MSI	10.70%	36.80%	NS
MMR expression loss	10.70%	36.80%	NS
KRAS mutations	25.00%	47.40%	NS
TP53 surexpression	60.70%	21.10%	0.0090[1]
MUC5AC surexpression	21.40%	47.40%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

to the 10 MSI-H tumors. All the MSS tumors showed normal MMR proteins expression. Hence, immunohistochemical analysis had 100% sensitivity for the detection of tumors with high MSI. Of note, germline deleterious mutations in *MMR* genes had been reported in our previous studies[14,24] in 4 patients with MSI-H CRC tumors and MMR protein expression loss.

TP53 protein expression analysis

TP53 positive nuclear immunostaining was observed in the CRC tumors from 21 (21/51, 41%) patients. No significant association has been detected between TP53 overexpression and selection criteria (Amsterdam or Bethesda) or the presence of relatives with CRC (Table 2). Overall, no association has been detected between TP53 overexpression and the different clinical parameters, including age at tumor diagnosis, gender, TNM stage or tumor location (Table 3). All but one (20/21, 95.2%) of the CRC tumors with TP53 overexpression were MSS and showed normal MMR proteins expression. The remaining tumor was of MSI-H phenotype associated to a combined MLH1 and PMS2 proteins expression loss, suggesting an MMR deficiency. On other hand, we have noted a close correlation between *TP53* mutations and TP53 protein level (P = 0.0090) (Table 5), as previously reported[25,26]. The absence of mutation in the 4 tumors overexpressing TP53 may be due to a lack of sensibility of the utilized sequencing technique, which requires greater than 15%-20% of neoplastic cells burden in the analysed specimens. In addition, mutations may be located outside the screened exons (exons 4-9), which represent less than 5% of the *TP53* detected mutations[27].

Expression analysis of MUC5AC

Abnormal MUC5AC expression was identified in 15 CRC tumors (15/51, 29.41%), 6 of them showed mucinous colloid component ⩾ 50%. In 3 tumors, the stained area was limited to the focal glands. MUC5AC expression was significantly associated to the presence of personal and family history of CRC (P = 0.039) (Table 2). It is very interesting to note that abundant MUC5AC expression was seen in the tumor of 3 HNPCC subjects with deleterious germline *MMR* mutations[14]. However, we didn't detect any other significant association between MUC5AC expression and clinico-pathological characteristics (Table 6). Interestingly, MUC5AC expression was significantly associated to MSI phenotype and MMR proteins expression loss (P = 0.0039) (Table 6). In contrast, no significant association was detected between MUC5AC expression and *TP53* or *KRAS* genes mutations (Table 6).

TP53 mutations analysis

The *TP53* mutation analysis was possible in the CRC

Table 6 Comparison of mucin 5AC expression as a function of tumoral parameters

	MUC5AC expression (n = 15)	Absence of MUC5AC expression (n = 36)	P
MSI phenotype (n = 10)	46.70%	8.30%	0.0039[1]
MMR expression loss (n = 10)	46.70%	8.30%	0.0039[1]
TP53 mutations (n = 28)	40.00%	68.80%	NS
TP53 overexpression (n = 21)	33.30%	44.40%	NS
KRAS mutations (n = 16)	26.70%	33.30%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

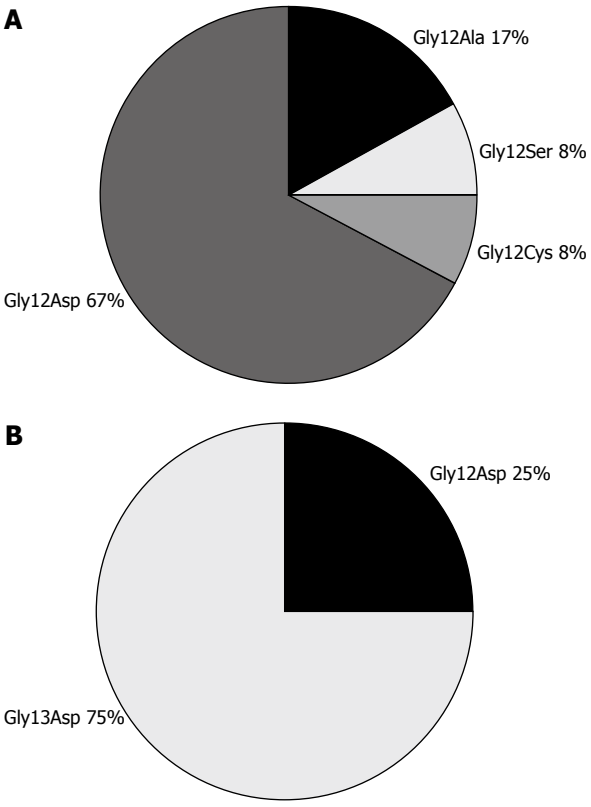

Figure 1 *KRAS* mutation spectrum as a function of tumoral microsatellite instability phenotype. A: Microsatellite stable (MSS); B: Microsatellite instability-high (MSI-H).

tumors of 47 patients. In total, a deleterious somatic mutation has been detected in 28 patients (28/47, 59.6%). Overall, there were no significant association of *TP53* mutations with Bethesda criteria, cancer family history (Table 2) or patient's clinical and histo-pathological data (Table 3). Particularly, we didn't detect any association between the presence of *TP53* mutations and tumor site (P = 0.0658) (Table 3). This was in contrast with data reported by other groups which showed that *TP53* mutations where more frequent in left-sided and rectal tumors[28-30]. A *KRAS* somatic mutation was identified in 7 (7/28, 25%) of the CRC tumors with *TP53* mutations (Table 5). All these mutations were G>A transitions

Table 7 Comparison *KRAS* somatic mutations as a function of tumoral parameters

	Presence of *KRAS* mutations (n = 16)	Absence of *KRAS* mutation (n = 35)	P
MSI	25.00%	17.10%	NS
MMR expression loss	25.00%	17.10%	NS
TP53 mutations	46.70%	65.60%	NS
TP53 overexpression	31.30%	45.70%	NS
MUC5AC overexpression	25.00%	31.40%	NS

NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

in codon 12 (5 mutations were p.Gly12Asp and 1 was p.Gly12Ser) and no mutation has been detected in codon 13.

KRAS somatic mutations

A *KRAS* mutations was identified in 16 (16/51, 31.5%) of all the CRC tumors. There was no significant association of *KRAS* mutations with Bethesda criteria, cancer family history (Table 2) and patient's clinical and histo-pathological data (Table 3). In addition, no significant association has been detected between *KRAS* mutations and the other tumoral parameters (Table 7). However, the mutation spectra same to be different between MSS and MSI tumors and more varied mutations have been detected in MSS tumors (Figure 1). Some amino acid changes were detected only in MSS tumors (Figure 1). Whereas, the *KRAS* mutation p.Gly13Asp have been detected only in MSI-H tumors in the absence of *TP53* mutations or TP53 overexpression (Figure 1).

BRAF mutations

The *BRAF* activating mutation c.1796A>T, p.Val600Glu was found in only 1 (1/51, 2%) stage Ⅱ non-mucinous and non-invasive tumor of the proximal colon of a 79 years old man with no cancer family history. This mutation was shown to be specific to sporadic CRC tumors due to *MLH1* promoter hypermethylation and absent in CRC tumors with MSS phenotype[31] and patients with *MLH1* or *MSH2* germline mutations[11]. Because we didn't examine the entire *BRAF* gene, we cannot rule out the presence of other mutations in the remaining CRC tumors. This tumor showed peritumoral lymphatic reaction, MSS phenotype, normal MMR proteins expression and abnormal MUC5AC expression. In addition, no somatic mutations in *KRAS* or *TP53* genes or overexpression of TP53 protein were detected in this tumor. According to some authors, this tumoral phenotype characterized CRC tumors arising from serrated polyps[32].

CTNNB1 mutations

Only 1 putative pathogenic somatic mutation was detected in an MSI-H CRC tumor (1/51, 2%). The change was a typical missense mutation causing alteration of serine at codon 45 (c.134C>T, p.Ser45Phe). This patient

was operated of TNM stage Ⅱ well differentiated and mucinous adenocarcinoma of the sigmoid at 36 years of age and didn't have any relatives with cancer. Tumor analysis detected a p.Gly13Asp *KRAS* mutation and a combined MLH1 and PMS2 proteins expression loss. These findings were in accordance with the specific affinity of *CTNNB1* mutation to MSI-H CRC tumors[5,33]. In addition, normal TP53 and MUC5AC proteins expression was detected in this tumor in the absence of *TP53* or *BRAF* somatic alterations. According to Young *et al*[34], this tumoral phenotype characterises CRC in Lynch syndrome, highlighting the presence of this syndrome in this patient.

MLH1 promoter methylation

Further analysis of *MLH1* promoter methylation status in the tumor of 4 patients showing MSI-H phenotype and MLH1 protein expression loss in the absence of *MLH1* deleterious somatic mutation by MLPA or sequencing, did not detect aberrant methylation discarding the hypothesis of sporadic cancers due to epigenetic inactivation of *MLH1* gene.

DISCUSSION

The detection of subjects at high risk of CRC remains problematic. It was essentially based on the family history of patients. Nevertheless, MSI testing and MMR protein expression analysis still the major screening tool for identifying HNPCC. In the present report, we have studied the phenotype and the genetic characteristics of the CRC tumors of 51 Tunisian non related patients selected according to the revised Bethesda criteria in order to compare the tumor phenotype due to MMR deficiency with somatic alterations in genes implicated in CRC tumorigenesis. Our aim was to define for each tumor the pathway of carcinogenesis and to identify new tumoral markers which may help in the diagnosis of CRC susceptibility and easy to use in medical practice. Clinical data analysis showed that CRC was essentially right sided in patients with first or second degree CRC relatives, whereas CRC was mostly distal (left colon and rectum) in patients without cancer family history. These findings are in accordance with data published[35,36]. As expected, genetic characteristics analysis of the 51 tumors showed that MSI phenotype and MMR expression loss were significantly associated to the presence of a CRC family history ($P < 0.001$). *TP53* mutations have been detected in 59.6% of the analysed patients. This finding was in agreement with previous studies in CRC, which reported *TP53* mutation frequencies between 50% and 70%[28-30]. Our study shows statistically inverse relationships between MSI and *TP53* alterations in CRC ($P = 0.0335$). This finding was in accordance with that reported by Samowitz *et al*[29]. This data highlights the hypothesis that MMR deficient CRC tumors evolve through a pathway that is independent of *TP53* gene. *KRAS* mutations were identified in 31.5% of all CRC tumors. This is consistent with previous reports that have identified *KRAS* mutations in 30%-45% of CRC tumors[8,29,37]. No significant association had been detected between MSI phenotype and *KRAS* alterations. However, the mutation spectrum was different between MSS and MSI-H tumors. In spite of our reduced number of tumors this finding was in accordance with that reported[8]. On the other hand, abnormal MUC5AC expression was found to be significantly associated to MSI phenotype ($P = 0.0039$) and CRC personal and family history ($P = 0.039$). In contrast, no significant association was detected between MUC5AC expression and *KRAS* or *TP53* genes mutations.

In conclusion, we suggest that MUC5AC expression analysis of CRC tumors may be useful in the screening of patients with high risk of CRC.

ACKNOWLEDGMENTS

This study was the result of collaboration between the Universities of Tunis-El Manar and Lille 2. We are indebted to all family members who agreed to attend our study. We also thank Dr Trabelsi O, Gastroenterologist in Tunis, Tunisia, for providing us with families, Dr Wacrenier A, Department of Pathology of the CHRU of Lille, France, for her help in immunohistochemistry of MMR proteins, and the "Plateau Commun de Biologie Moléculaire du Centre de Biologie-Pathologie" of the CHRU of Lille.

COMMENTS

Background

This paper tend to make correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC) on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

Research frontiers

Authors have screened 51 CRC tumors containing mixed hereditary non-polyposis colorectal cancer (HNPCC) and sporadic CRC Tunisian cases for somatic changes in *KRAS*, *BRAF* and *CTNNB1*, for microsatellite instability, for expression of mismatch repair (MMR) and mucin (MUC) 5AC, for mutation and expression of *TP53* and for *MLH1* promoter hypermethylation. They have also compared these molecular findings with clinical, pedigree and pathological data, regardless of age, personal or family history of cancer, and tumor characteristics.

Innovations and breakthroughs

In this study, authors report for the first time in Tunisia the value of various histo-pathologic features and somatic molecular changes in distinguishing patients with HNPCC from those with sporadic colorectal cancer in the aim to identify new tumoral markers of colorectal cancer susceptibility easy to use in the design of diagnostic, therapeutic and preventive strategies in Tunisia.

Applications

MUC5AC expression was significantly associated to the presence of the presence of familial CRC, microsatellite instability and MMR expression loss. This finding suggests that MUC5AC expression analysis may be useful in the screening of patients with high risk of CRC in Tunisia.

Peer review

Screening the subjects at risk of CRC is important. In this manuscript, the authors retrospectively reviewed the histo-pathological features, molecular changes and family history of 51 Tunisian CRC patients. Among many genetic and clinical variables, MUC5AC expression was concluded to be useful in the screening of patients at high risk of CRC susceptibility.

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P- Reviewers Li YY, Rodriguez DC **S- Editor** Gou SX
L- Editor A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5295

World J Gastroenterol 2013 August 28; 19(32): 5295-5301
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Investigation of genome instability in patients with non-alcoholic steatohepatitis

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Received: March 7, 2013 Revised: May 17, 2013
Accepted: June 1, 2013
Published online: August 28, 2013

Abstract

AIM: To evaluate the occurrence of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) in the mitogen-stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH).

METHODS: The study was performed in 25 (9 females, 16 males) patients newly diagnosed with NASH, and 25 healthy subjects of similar ages and genders were used as a control group. None of the controls was known to be receiving any drugs for medical or other reasons or using alcohol. Hepatosteatosis was further excluded by abdominal ultrasound imaging in the control group. The numbers of MN, NPBs and NBUDs scored in binucleated (BN) cells were obtained from the mitogen-stimulated lymphocytes of patients and control subjects. Statistical comparisons of the numbers of BN cells with MN, NPBs and NBUDs and ages between the patients with NASH and control subjects were performed.

RESULTS: The mean ages of the patients and the control group were 41.92 ± 13.33 and 41.80 ± 13.09 years ($P > 0.05$), respectively. The values of the mean body mass index (BMI), HOMA-IR, hemoglobin, creatinin, aspartate aminotransferase, alanine aminotransferase, triglyceride, high density lipoprotein, and low density lipoprotein were 31.19 ± 4.62 kg/m^2 *vs* 25.07 ± 4.14 kg/m^2, 6.71 ± 4.68 *vs* 1.40 ± 0.53, 14.73 ± 1.49 g/dL *vs* 14.64 ± 1.30 g/dL, 0.74 ± 0.15 mg/dL *vs* 0.80 ± 0.13 mg/dL, 56.08 ± 29.11 U/L *vs* 16.88 ± 3.33 U/L, 92.2 ± 41.43 U/L *vs* 15.88 ± 5.88 U/L, 219.21 ± 141.68 mg/dL *vs* 102.56 ± 57.98 mg/dL, 16.37 ± 9.65 mg/dL *vs* 48.72 ± 15.31 mg/dL, and 136.75 ± 30.14 mg/dL *vs* 114.63 ± 34.13 mg/dL in the patients and control groups, respectively. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs, which were scored using the CBMN cytome assay on PHA-stimulated lymphocytes, were evaluated in the patients with NASH and control group. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$).

CONCLUSION: The increased numbers of MN, NPBs and NBUDs observed in the lymphocytes obtained from patients with NASH may reflect genomic instability.

Key words: Non-alcoholic steatohepatitis; Micronucleus; Nucleoplasmic bridges; Nuclear buds

Core tip: We aimed to evaluate the micronucleus, nucleoplasmic bridges and nuclear buds in the mitogen-

stimulated lymphocytes of patients with non-alcoholic steatohepatitis (NASH). Genomic instability may be a stage in the development of hepatic carcinogenesis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma (HCC). Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there is high genomic instability in their mitogen-stimulated lymphocytes. Further prospective studies are needed to further clarify this topic, especially among patients with HCC, cirrhosis and NASH.

Karaman H, Karaman A, Donmez-Altuntas H, Bitgen N, Hamurcu Z, Oguz A, Karakukcu C. Investigation of genome instability in patients with non-alcoholic steatohepatitis. *World J Gastroenterol* 2013; 19(32): 5295-5301 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5295.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5295

INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. Non-alcoholic fatty liver (NAFL) is characterized by steatosis without inflammation and fibrosis[1]; its prevalence is 10%-30% in adults[2]. NASH is a major cause of so-called cryptogenic liver cirrhosis[3] and cause hepatocellular carcinoma[4,5].

The use of the cytokinesis-blocked micronucleus (CBMN) assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk[6]. The CBMN assay allows once-divided cells to be recognized by their binucleated (BN) cell appearances after the inhibition of cytokinesis by cytochalasin B[7]. This method was initially proposed for the evaluation of the micronucleus (MN) in BN cells. However, the CBMN assay has more recently been considered a multipurpose test because it can analyze the proliferation index (a measure of cytostasis), cell death (a measure of cytotoxicity) and DNA damage[8-10]. It is often called a cytome assay[11,12]. The events of DNA damage are scored specifically in once-divided BN cells. The frequency of BN cells with MN, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) provides a measure of genome instability or DNA damage. MN is formed through different processes, such as chromosome breakage or complete chromosome loss that lags behind anaphase in cell division. NPBs originate from asymmetrical chromosome rearrangements and/or telomere end fusions. NBUDs are considered biomarkers of gene amplification[9,12].

In this study, our objective was to determine the spontaneous number of MN, NPBs and NBUDs in the phytohemagglutinin (PHA)-stimulated lymphocytes of patients with NASH.

MATERIALS AND METHODS

Patients and controls

This study was conducted between August 2012 and September 2012 in Kayseri Educational and Research Hospital Department of Gastroenterology. Written informed patient consent was obtained from each patient before the procedure, and the study was approved by the Ethics Committee of Kayseri Educational and Research Hospital. The study was performed on 25 (9 females, 16 males) patients newly diagnosed with NASH and on 25 healthy controls of similar ages and genders. None of the participants was known to be receiving any drugs for medical or other reasons or using alcohol. In addition, hepatosteatosis was excluded by abdominal ultrasound imaging in the control group.

Inclusion and exclusion criteria

Each subject in the NASH group had a history of chronic serum alanine aminotransferase (ALT) elevation, which was defined as an ALT > 40 U/L that occurred on two separate occasions separated by at least 3 mo (90 d). No patients or control subjects had an alcohol habit. The subjects also underwent a work-up for other causes of chronic hepatitis of unknown etiology, including serological evaluation for alpha-1-antitrypsin, hepatitis B surface antigen, hepatitis C antibody, copper, ceruloplasmin, antinuclear antibody, anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and total immunoglobulin G. None of the patients, controls, or any of their first degree relatives had diabetes mellitus. Subsequently, the NASH subjects underwent a standard-of-care liver biopsy to identify the etiology and severity of NASH. To be included in the study, the biopsy had to show macrovesicular fat in a minimum of 5% of the hepatocytes, the absence of other etiologies identifying the presence of fat, and a pattern of injury consistent with NASH, as determined by a pathologist[13].

Our patients and control subjects were asked about and examined for conditions affecting MN frequency, including malnutrition, occupational or environmental exposure to known genotoxic agents, smoking, and tea or coffee drinking. None of the patients were receiving medication.

Body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), aspartate aminotransferase (AST), ALT, triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), hemoglobin (Hb) and creatinin were measured or calculated for both groups.

Lymphocyte cultures for CBMN assay

Three milliliter blood samples were collected in heparinized tubes from the antecubital vein after informed consent had been obtained from all patients and control subjects. Approximately 0.4 mL of heparinized whole blood samples was cultured for 72 h at 37 ℃ in 5 mL of

Table 1 Demographic and laboratory parameters of the patient and control groups

Parameter	*n*	mean	SD	*P*
Age (yr)				0.977
Patient	25	41.92	13.33	
Control	25	41.80	13.09	
BMI (kg/m^2)				0.001
Patient	25	31.19	4.62	
Control	25	25.07	4.14	
HOMA-IR				0.001
Patient	25	6.71	4.68	
Control	25	1.40	0.53	
Hb (g/dL)				0.80
Patient	25	14.73	1.49	
Control	25	14.64	1.30	
Creatinine (mg/dL)				0.12
Patient	25	0.74	0.15	
Control	25	0.80	0.13	
AST (U/L)				0.001
Patient	25	56.08	29.11	
Control	25	16.88	3.33	
ALT (U/L)				0.001
Patient	25	92.20	41.43	
Control	25	15.88	5.88	
TG (mg/dL)				0.001
Patient	25	219.21	141.68	
Control	25	102.56	57.98	
HDL (mg/dL)				0.52
Patient	25	46.37	9.65	
Control	25	48.72	15.31	
LDL (mg/dL)				0.02
Patient	25	136.75	30.14	
Control	25	114.63	34.13	

BMI: Body mass index; HOMA-IR: Homeostasis model assessment insulin resistance; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Hb: Hemoglobin.

peripheral blood karyotyping medium that was supplemented with 1.5% phytohemagglutinin-M to stimulate T-lymphocytes (all from Biological Industries, Kibbutz Beit Haemek, Israel).

In our study, two parallel cultures were prepared simultaneously for each patient and control subject to determine their intra-individual differences. Different slides of two parallel cultures were prepared[14].

Forty-four hours after the initiation of the cultures, the cells were blocked from entering cytokinesis by the addition of cytochalasin-B to each culture tube at a final concentration of 3 μg/mL (Sigma-Aldrich)[14]. The cultures were stopped at 72 h after initiation, treated with hypotonic solution (0.1 mol/L KCl) for 4 min and fixed in two changes of methanol:acetic-acid (3:1)[15]. The fixed cells were spread onto glass slides and stained with 5% Giemsa (Merck) in Sorensen's buffer for 10 min.

CBMN cytome assay

Different slides of two parallel cultures from each patient and control subject were prepared and evaluated. All slides were evaluated blindly using a Nikon Alphaphot-2 light optical microscope. For each sample (patient and control subject), 1000 BN cells were scored for the numbers of micronucleus (MN), NPBs, and nuclear buds (BUDs) in the lymphocytes of the patients and control subjects. The published criteria for the determinations of BN cells, MN, NPBs and NBUDs were followed[12].

Statistical analysis

Statistical comparisons of the number of BN cells with MN, NPBs, NBUDs and the ages of the patients with NASH with those of the control subjects were performed using a non-parametric Mann-Whitney *U* test for two independent samples. Spearman's rho correlation analysis was used to determine the relationships among age and the numbers of MN, NPBs and NBUDs.

RESULTS

The mean ages of the patients and control group were 41.92 ± 13.33 and 41.80 ± 13.09 years, respectively ($P > 0.05$). The demographic and laboratory parameters of the patient and control groups are shown in Table 1. The total numbers and frequencies of BN cells with MN, NPBs and NBUDs scored using a CBMN cytome assay in PHA-stimulated lymphocytes from patients with NASH are shown in Table 2, and those of the control group are shown in Table 2. We found significantly higher numbers of MN, NPBs and NBUDs in the BN cells of patients with NASH than in those of the control subjects (21.60 ± 9.32 *vs* 6.88 ± 3.91; 29.28 ± 13.31 *vs* 7.84 ± 3.96; 15.60 ± 5.55 *vs* 4.20 ± 1.63, respectively, $P < 0.0001$) (Table 3).

DISCUSSION

In the present study, the numbers of MN, NPBs and NBUDs in the lymphocytes of patients with NASH showed a significant increase compared to the control group. Considering the lack of data in the literature related to our values obtained for MN, NPBs and NBUDs, it was not possible to make direct comparisons with other studies. However, the formation of nuclear anomalies, including MN, NPBs and NBUDs, have previously been reported as events commonly observed in the early stages of carcinogenesis[6]. Therefore, we believe that the increased presence of DNA damage biomarkers, including MN, NPBs and NBUDs, in the lymphocytes of patients with NASH may be associated with an increased risk of developing liver cancer.

Oxidative stress, genetic defects in cell cycle checkpoints, defects in DNA repair genes or environmental/dietary factors can each cause the formation of MN *via* chromosomal rearrangements, altered gene expressions or aneuploidy, all of which are associated with a chromosome instability phenotype that is observed primarily in cases of cancer[16,17].

Some previous studies have discussed the association between the induction of MN and the development of cancer. In untreated cancer patients and in subjects with cancer-prone congenital diseases, such as Bloom

Table 2 Total numbers and frequencies of binucleated cells with micronucleus, nucleoplasmic bridges and nuclear buds scored using the cytokinesis-blocked micronucleus cytome assay in phytohemagglutinin-stimulated lymphocytes from patients with non-alcoholic steatohepatitis and the control subjects

ID	Age (yr)	Sex	No. of MN in BN cells[1]	Distribution of BN cells with					No. of BN cells with NPBs	No. of BN cells with NBUDs
				1MN	2MN	3MN	4MN	5MN		
Patients with non-alcoholic steatohepatitis										
1	45	M	19	15	-	-	1	-	25	19
2	60	M	24	19	1	1	-	-	46	11
3	50	M	33	24	1	1	2	-	33	22
4	66	M	41	39	1	-	-	-	20	22
5	22	M	8	8	-	-	-	-	32	17
6	43	M	33	29	2	-	-	-	25	14
7	36	F	14	14	-	-	-	-	21	17
8	51	F	9	9	-	-	-	-	16	10
9	35	M	21	16	-	-	-	1	25	14
10	42	M	11	11	-	-	-	-	12	15
11	30	F	28	24	2	-	-	-	50	20
12	22	M	11	7	2	-	-	-	17	15
13	31	M	12	10	1	-	-	-	21	16
14	41	M	15	13	1	-	-	-	18	25
15	33	M	10	10	-	-	-	-	30	10
16	44	F	22	17	1	1	-	-	58	21
17	47	M	20	13	2	1	-	-	40	17
18	20	M	25	16	1	1	-	1	14	10
19	22	M	24	20	-	-	1	-	32	8
20	46	F	37	27	2	2	-	-	25	10
21	68	F	36	26	5	-	-	-	63	28
22	48	M	17	15	1	-	-	-	28	6
23	60	M	22	18	-	-	1	-	36	19
24	45	F	24	20	2	-	-	-	25	14
25	41	M	24	19	1	1	-	-	20	10
The control subjects										
1	45	M	5	5	-	-	-		11	3
2	60	M	8	6	1	-	-		10	5
3	50	M	7	7	-	-	-		6	5
4	66	M	11	9	1	-	-		12	6
5	22	M	2	2	-	-	-		2	4
6	43	M	6	4	1	-	-		1	4
7	36	F	3	3	-	-	-		9	4
8	51	F	9	5	2	-	-		5	3
9	35	M	4	2	1	-	-		4	1
10	42	M	6	6	-	-	-		7	5
11	30	F	8	8	-	-	-		1	4
12	22	M	2	2	-	-	-		8	4
13	31	M	2	2	-	-	-		10	5
14	41	M	7	5	1	-	-		16	8
15	33	M	7	7	-	-	-		12	6
16	44	F	11	8	-	1	-		5	6
17	47	M	9	7	1	-	-		4	2
18	20	M	1	1	-	-	-		8	4
19	22	M	4	4	-	-	-		9	1
20	46	F	11	7	2	-	-		8	3
21	65	F	17	15	1	-	-		9	5
22	48	M	12	10	1	-	-		14	6
23	60	M	6	6	-	-	-		13	3
24	45	F	11	9	1	-	-		5	3
25	41	M	3	3	-	-	-		7	5

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Total number of MN: (1MNX1) + (2MNX2) + (3MNX3) + (4MNX4) + (5MNX5). M: Male; F: Female.

Syndrome, an increased frequency of MN has been shown[16,18]. Clinical chemoprevention trials on oral premalignancies have used MN in the oral mucosa as a surrogate endpoint of cancer[19,20]. Another piece of corroborating evidence concerning the association between the MN frequency and the development of cancer is the correlation between genotoxic MN-inducing agents, such as ionizing and ultraviolet radiation, and carcinogenesis[21,22].

Bonassi *et al*[6] evaluated the MN frequency in a total of 6718 subjects selected from the database of Human

Table 3 The numbers of micronucleus, nucleoplasmic bridges and nuclear buds in phytohemagglutinin-stimulated lymphocytes from patients and controls (means ± SD)

Group	Age (yr)	No. of MN in BN cells	No. of BN cells with NPBs	No. of BN cells with NBUDs
Patients (n = 25)	41.92 ± 13.33	21.60 ± 9.32	29.28 ± 13.31	15.60 ± 5.55
Controls (n = 25)	41.80 ± 13.09	6.88 ± 3.91	7.84 ± 3.96	4.20 ± 1.63
P value	0.977	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
Z value	0.029	5.413	5.953	6.016

The numbers of micronucleus (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored on 1000 binucleated (BN) cells per subject. [1]Patients with non-alcoholic steatohepatitis exhibited statistically higher numbers of MN, NPBs and NBUDs in 1000 BN cells than controls, according to the two-tailed nonparametric Mann-Whitney *U*-test for the comparison of the means of independent variables.

Micronucleus Projects, and they followed the subjects for cancer incidence or mortality. After a median duration for follow-up of 8 years, 219 incident cancers and 56 cancer deaths were detected. The subjects in the medium/high MN frequency groups demonstrated a significant correlation between overall cancer incidence and MN frequency (the P values were 0.001 and 0.03, respectively). The risks associated with specific cancer sites were also tested. All cancer sites except for the hepatobiliary and pancreas primaries (RR = 0.163; 0.27-1.44) were shown to have higher relative risks in the medium/high MN groups. The most prominent risk increase was found for bladder and kidney cancers (RR = 8.23; 1.08-63.0). The group concluded that MN frequency in PBL is predictive of cancer risk[6].

The MN technique provides a convenient and reliable index of both chromosome breakage and chromosome loss[18,23]. No studies have been conducted regarding MN formation in the lymphocytes of patients with NASH. In our study, a significant increase in the number of MN was found in the stimulated lymphocytes of patients with NASH. These results strongly support the theory that genomic impairment is elevated in the lymphocytes of patients with NASH.

In addition, the number of MN may be related to other factors, such as micronutrients (folate and riboflavin concentration), occupational or environmental exposure, genetic polymorphisms, lifestyle, smoking and tea or coffee drinking[24-26]. Our patients and control subjects were free from any conditions affecting their MN frequency, such as malnutrition, occupational or environmental exposure. The smoking, tea and coffee habits of the patients and control subjects were similar.

It has been reported that patients who are affected by familiar cutaneous malignant melanoma[27] or cancer-prone congenital diseases, *e.g.*, Bloom syndrome or ataxia telangiectasia, have abnormally high MN frequencies[28]. Moreover, Karaman *et al*[29] observed a significant increase in the MN levels of the lymphocytes of patients with colorectal adenocarcinomas and neoplastic polyps. Hamurcu *et al*[15] showed a clear increase in the frequency of MN in the peripheral lymphocytes of untreated cancer patients. In our previous study, we reported high MN, NPB and NBUD ratios in patients with ulcerative colitis[30]. Additionally, increased MN frequency has been reported in patients with diseases with high cancer risks, such as acromegaly[31] and polycystic ovary syndrome[32].

There are some reports about NASH-related hepatocellular carcinoma (HCC)[4,33-35]. Takuma *et al*[36] reviewed the literature and reported 105 cases (11 of them were their patients) of NASH-associated HCC. They reported that patients with non-cirrhotic NASH may be a high-risk group for HCC. Our results support this suggestion; although none of the patients had liver cirrhosis in our study, there was high genomic instability in their mitogen-stimulated lymphocytes.

Further studies are required to understand the importance of MN, NPBs and NBUDs on NASH-related genomic damage and hepatocellular carcinoma.

COMMENTS

Background

Non-alcoholic steatohepatitis (NASH) is an underdiagnosed liver disease characterized by steatosis and necroinflammation with hepatocyte injury (ballooning), with or without fibrosis. NASH is a major cause of so-called cryptogenic liver cirrhosis and can result in hepatocellular carcinoma. The use of the cytokinesis-blocked micronucleus assay on peripheral blood lymphocytes is one of the most well-validated cytogenetic tests for measuring DNA damage, genome instability and cancer risk. Authors evaluated the risk of genomic instability in patients with NASH in this study.

Research frontiers

The micronucleus (MN) technique provides a convenient and reliable index of both chromosome breakage and chromosome loss. The technique is simple and inexpensive, but it provides important knowledge about genomic instability and DNA damage.

Innovations and breakthroughs

This is the first study that investigates DNA damage in patients with NASH using this method.

Applications

Patients with NASH show genomic instability, but further studies investigating genomic instability in patients with cirrhosis and hepatocellular carcinoma are necessary.

Terminology

MN is formed through several different processes, such as chromosome breakage or complete chromosome loss, that lag behind anaphase in cell division. Nucleoplasmic bridges originate from asymmetrical chromosome rearrangements and/or telomere end fusions; nuclear buds are considered biomarkers of gene amplification.

Peer review

This is a good study that investigates the micronucleus frequency in patients with NASH. A suggestion to the authors is that the patients with high micronucleus ratios should be followed to observe whether they develop hepatocellular carcinoma.

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P- Reviewers Chamberlain SM, Xu J **S- Editor** Wen LL
L- Editor A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5302

World J Gastroenterol 2013 August 28; 19(32): 5302-5308
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

High level of preoperative carbohydrate antigen 19-9 is a poor survival predictor in gastric cancer

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Author contributions: Choi AR and Park JC contributed equally to this work; Choi AR, Park JC and Chung JB designed the study and performed the majority of experiments; Kim JH, Shin SK, Lee SK and Lee YC identified and recruited patients for the study and undertook the experiments; Kim JH and Shin SK were responsible for data collection, data extraction, data interpretation and manuscript drafting; Lee SK and Lee YC performed bioinformatic analysis of the sequencing data and performed statistical analyses; Choi AR and Park JC co-wrote the manuscript and were also involved in editing the manuscript; all authors reviewed and agreed on the final version.

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Received: May 16, 2013 Revised: July 10, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

AIM: To assess the clinical significance and the prognostic value of preoperative serum carbohydrate antigen 19-9 (CA 19-9) level in gastric cancer.

METHODS: Between January 2005 and December 2006, 1960 patients underwent surgery for histologically confirmed gastric cancer. Of these, 163 patients had elevated serum levels of CA 19-9 preoperatively, and 1628 patients had normal serum levels of CA 19-9 preoperatively. For this study, 325 patients were selected from the group of 1628 patients by age, sex, and cancer stage to serve as controls. Statistically significant differences in survival rates were calculated using the log-rank test. A *P* value less than 0.05 was considered statistically significant and was determined using SAS software.

RESULTS: The baseline characteristics showed some differences between the two groups with regard to histology. Overall survival (OS) in the elevated and non-elevated group was 37.90 and 68.67 mo, respectively ($P < 0.001$). N stage ($P = 0.001$) was a significant predictor of disease-free survival by multivariate analysis. Also, N stage ($P < 0.001$), and the presence of peritoneal metastasis ($P < 0.001$) remained independent factors in predicting OS by multivariate analysis. Additionally, preoperative serum CA 19-9 levels were significantly associated with OS in univariate ($P = 0.009$) and multivariate ($P = 0.021$) analyses.

CONCLUSION: Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

Key words: Gastric cancer; Carbohydrate antigen 19-9; Disease-free survival; Overall survival

Core tip: The exact functions of preoperative carbohydrate antigen (CA) 19-9 in stomach cancer have yet to be uncovered. We sought to assess the clinical significance of preoperatively high levels of CA 19-9 in patients with gastric cancer and aimed to investigate the relationship between serum levels of CA 19-9 and disease-free survival and overall survival (OS). We conclude that OS in gastric cancer patients with elevated CA 19-9 levels was lower than that in patients with non-elevated levels. Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

Choi AR, Park JC, Kim JH, Shin SK, Lee SK, Lee YC, Chung JB. High level of preoperative carbohydrate antigen 19-9 is a poor survival predictor in gastric cancer. *World J Gastroenterol* 2013; 19(32): 5302-5308 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5302.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5302

INTRODUCTION

Gastric cancer is one of the most common malignancies and the cause of many cancer-related deaths worldwide. Although a single tumor marker is limited for the diagnosis of cancer, it can be used in various clinical aspects, including assessment of clinical status, monitoring of treatment response, prediction of prognoses, and as a surveillance marker for recurrence[1-6]. Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 are tumor markers that are commonly used for the early diagnosis and prognostic evaluation of gastric cancer[7-9], potentially reflecting tumor biology[10]. Additionally, the relatively new marker cancer antigen, CA 72-4 provides prognostic information in gastric cancer[11,12].

Recent clinical studies have shown that CEA and CA 19-9 are recognized as poor prognostic factors for gastric cancer[13-15] and are related to its recurrence[6,14]. The prognostic relevance of such tumor markers in patients with gastric cancer is not comparable with those markers used in other carcinomas[5,16-18]. Specifically, CA 19-9 has been reported to be elevated in certain forms of gastric cancer[11,19,20]. However, because little research on the prognoses of gastric cancer patients with elevated preoperative CA 19-9 levels has been performed, the clinical significance of preoperative CA 19-9 levels has not been fully verified[5,13].

Therefore, it is important to interpret the prognostic value of CA 19-9 levels in patients with gastric cancer, especially in patients anticipating surgery for postoperative survival. Thus, we sought to assess the clinical significance of preoperatively high levels of CA 19-9 in patients with gastric cancer and aimed to investigate the relationship between serum levels of CA 19-9 and disease-free survival (DFS) and overall survival (OS).

MATERIALS AND METHODS

Patients

Between January 2005 and December 2006, 1960 patients underwent surgery for histologically confirmed gastric cancer at Severance Hospital, Seoul, South Korea. Sixty-nine patients who did not have preoperative serum CA 19-9 were excluded. Of the remaining 1891, 163 patients had elevated serum levels of CA 19-9 preoperatively, and 1628 patients had normal serum levels of CA 19-9 preoperatively. For this study, 325 patients were selected from the group of 1628 patients by age, sex, and cancer stage to serve as controls. A separate group of 488 patients who received surgery as a treatment modality for confirmed gastric cancer was included and analyzed retrospectively in this study.

Classification of gastric cancers

The endoscopic findings of early gastric cancer were classified according to the criteria of the Japanese Research Society for Gastric Cancer (JRSGC) as follows: elevated (types I or ⅡA), flat (type ⅡB), depressed (types ⅡC, ⅡC + Ⅲ, or ⅡA + ⅡC), and mixed. Advanced gastric cancers were categorized according to Borrmann's classification. Histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology from the JRSGC[21].

In addition, patients were classified into three groups, which were based on the location of the primary lesion: upper third, middle third, and lower third. The upper third-designated cancer developed in the gastric cardia and fundus, the middle third-designated cancer developed in the gastric body, and the lower third-designated cancer was found in the antrum and pylorus[22].

Treatment modalities

Surgical treatments were considered curative and palliative according to the Union for International Cancer Control criteria[23]. The standard surgical treatment was radical total or subtotal gastrectomy with D2 lymph node dissection in accordance with JRSGC rules[21]. Curative resection (R0) was defined as the absence of tumor either macroscopically or microscopically after surgery. In selected inoperable cases, palliative gastrectomy was performed when necessary.

Initial work-up and follow-up

A follow up period was started on January 1st, 2005 and ended on August 22th, 2008. Initial evaluation included complete medical history and physical examination, paying special attention to symptoms often associated with stomach cancer. Chest radiography and laboratory tests were performed, including complete blood cell count, blood urea nitrogen and creatinine levels, and liver function tests. Serum CA 19-9 concentrations were measured using a commercial chemiluminescent enzyme immunoassay with a normal upper limit of 37 U/mL[24]. Serum CA 19-9 levels were routinely measured immediately before surgery. The entire study population underwent esophagogastroduodenoscopy and computed tomography of the abdomen. After surgery, esophagogastroduodenoscopy, computed tomography of the abdomen and laboratory tests performed during the initial work-up were repeated at each follow-up visit.

Statistical analysis

This study was based on matched pair data considering age, sex and cancer stage. In the mixed model, comparisons between patients with elevated CA 19-9 levels and those with normal levels based on age, sex, cancer stage, and survival were performed. Categorical data were evaluated by the χ^2 test or Fisher's exact test, and all continuous variables were expressed as the median (range) and analyzed using the Mann-Whitney *U* test. Multivariate analysis of survival was performed using the Cox proportional hazards model. OS was defined from the date of surgery until death or the date of last follow-up. DFS was defined as the interval from the operation date to the date of confirming recurrence, death from any cause other than cancer, or last visiting date. Paired Kaplan-Meier

Table 1 Baseline characteristics with a comparison of patients with elevated serum carbohydrate antigen 19-9 levels and normal serum carbohydrate antigen 19-9 levels

Characteristic	Elevated group (*n* = 163)	Non-elevated group (*n* = 325)	*P* value
Sex			0.963
Male	111 (68.1)	222 (68.3)	
Female	52 (31.9)	103 (31.7)	
Mean age, yr (range)	60.70 ± 12.00 (29-84)	59.71 ± 12.40 (27-85)	0.295
Number of lesions			0.436
Single	145 (89.0)	281 (86.5)	
Multiple	18 (11.0)	44 (13.5)	
Size (cm)			
Horizontal	6.38 ± 3.51	5.78 ± 3.16	0.060
Vertical	5.15 ± 2.79	4.66 ± 2.60	0.053
Endoscopic findings			0.135
EGC			
Elevated	10 (6.1)	23 (7.1)	
Flat	3 (1.8)	15 (1.6)	
Depressed	10 (6.1)	18 (5.5)	
AGC			
Borrmann Ⅰ	9 (5.5)	8 (2.5)	
Borrmann Ⅱ	25 (15.3)	38 (11.7)	
Borrmann Ⅲ	89 (54.6)	203 (62.5)	
Borrmann Ⅳ	17 (10.4)	20 (6.2)	
Histology			0.033
Well differentiated	19 (11.7)	23 (7.1)	
Moderately differentiated	57 (35.0)	86 (26.5)	
Poorly differentiated	54 (33.1)	145 (44.6)	
Signet ring cell cancer	33 (20.3)	71 (21.9)	
Location			0.404
Lower	108 (66.3)	210 (61.6)	
Middle	16 (9.8)	49 (15.1)	
Upper	31 (19.0)	52 (16.0)	
Diffuse	8 (1.9)	14 (4.3)	
T stage			0.843
T0-2	55 (36.7)	117 (37.6)	
T3, 4	95 (63.3)	194 (62.4)	
N stage			0.908
N0, 1	79 (52.7)	162 (52.1)	
N2, 3	71 (47.3)	149 (47.9)	
Mean metastatic Lymph nodes (N)	9.88 ± 13.03	10.09 ± 11.94	0.893
TNM stage			> 0.999
0	1 (0.6)	2 (0.6)	
Ⅰ	26 (16.0)	52 (16.0)	
Ⅱ	21 (12.9)	42 (12.9)	
Ⅲ	57 (35.0)	114 (35.1)	
Ⅳ	58 (35.6)	115 (35.4)	
Operation			0.114
For radical	139 (85.3)	293 (90.2)	
For palliative	24 (14.7)	32 (0.8)	
Lymphovascular invasion			0.225
Positive	98 (76.0)	190 (70.1)	
Negative	31 (24.0)	81 (29.9)	
Peritoneal metastasis			0.899
Positive	10 (6.1)	19 (5.8)	
Negative	153 (93.9)	306 (94.2)	
Hepatic metastasis			0.853
Positive	5 (3.1)	11 (3.4)	
Negative	158 (96.9)	314 (96.6)	
Neoadjuvant chemotherapy			0.217
Positive	6 (3.7)	6 (1.8)	
Negative	157 (96.3)	319 (98.2)	
Mean serum CA 19-9 (range)	575.74 ± 518.09 (37.4-12800)	8.45 ± 8.42 (0-36.8)	< 0.001
Mean serum CEA (range)	6.00 ± 21.86 (0.01-260.27)	5.49 ± 17.30 (0.01-189.21)	0.777
Mean serum CA 72-4 (range)	9.31 ± 20.52 (0.33-164)	14.42 ± 68.61 (0.2-600)	0.376

Data are expressed as mean ± SD or *n* (%). AGC: Advanced gastric cancer; CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; EGC: Early gastric cancer; TNM: Tumor-node-metastasis.

curves and Cox regression analyses using robust standard error for survival analysis were performed. Statistically significant differences in survival rates were calculated using the log-rank test. A *P* value less than 0.05 was considered statistically significant and was determined using SAS software (version 9.1.3, SAS Institute Inc., Cary, NC, United States).

RESULTS

Patient characteristics

We compared the outcomes and clinicopathologic characteristics of 163 patients with elevated preoperative serum CA 19-9 levels (elevated group) with those of 325 patients with non-elevated preoperative serum CA 19-9 levels (non-elevated group), which are summarized in Table 1. Baseline characteristics did not show a significant statistical relationship between the two groups except for histology and serum CA 19-9 levels, which revealed a significantly higher proportion of less differentiated adenocarcinoma in patients with elevated preoperative serum CA 19-9 levels and mean serum CA 19-9 values of 575.74 ± 518.09 U/mL in the elevated group and 8.45 ± 8.42 U/mL in the non-elevated group. However, there were no significant differences in baseline characteristics with regard to other variables, such as sex, age, endoscopic findings, and other serum tumor markers. In 56 patients, surgery was performed as a palliative treatment. Of these, gastrojejunostomy was performed for bypass in 27 patients. Hepatic and peritoneal metastases were appraised by radiological findings, histological examination, and/or intraoperative observation.

Survival outcome according to preoperative CA 19-9 levels

The median OS was 58.433 mo (95%CI: 43.07-70.90). A significantly longer median OS was observed in the non-elevated group than in the elevated group (68.67 mo *vs* 37.90 mo, 95%CI: 25.07-56.13; $P < 0.001$; Figure 1A). Because the majority of patients died near the end of the study, the upper limit of the confidence interval in the non-elevated group was not calculated.

As survival curves for DFS did not reach 50% after Kaplan-Meier analysis, a median DFS was not defined. A longer DFS was seen in the non-elevated group than in the elevated group, but the difference was not statistically significant ($P = 0.099$; Figure 1B).

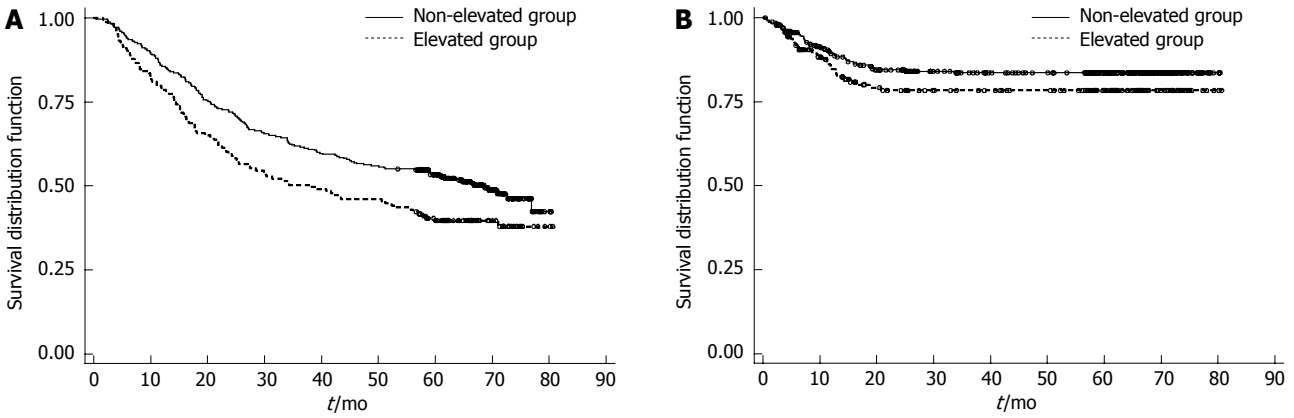

Figure 1 Kaplan-Meier curves. A: Overall survival, *P*-value by log-rank test < 0.001; B: Disease free survival in patients with elevated serum carbohydrate antigen 19-9 (CA 19-9) levels (n = 163) and those with normal serum CA 19-9 levels (n = 325), *P*-value by log-rank test = 0.099.

Table 2 Univariate and multivariate analysis of factors associated with disease-free survival

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Disease-free survival				
Age (< 60 *vs* ⩾ 60 yr)	0.737 (0.475-1.143)	0.737	0.620 (0.379-1.015)	0.053
Sex (male *vs* female)	1.140 (0.720-1.806)	0.575	0.777 (0.454-1.331)	0.359
Lesions (single *vs* multiple)	1.350 (0.746-2.446)	0.322	0.930 (0.458-1.892)	0.842
T staging (T 0-2 *vs* T 3, 4)	2.469 (1.442-4.228)	0.001	1.841 (0.961-3.527)	0.066
N staging (N 0, 1 *vs* N 2, 3)	4.069 (2.445-6.772)	< 0.001	2.993 (1.587-5.646)	0.001
Differentiation (well *vs* poorly)	1.378 (0.863-2.201)	0.179	1.419 (0.786-2.563)	0.246
Histology (adenocarcinoma *vs* signet ring cell cancer)	0.979 (0.573-1.674)	0.940	0.599 (0.315-1.142)	0.119
Lymphovascular invasion (negative *vs* positive)	3.054 (1.514-6.161)	0.002	1.15 (0.495-2.674)	0.745
Peritoneal metastasis (negative *vs* positive)	0.956 (0.301-3.034)	0.939	0.507 (0.069-3.704)	0.503
Hepatic metastasis (negative *vs* positive)	0.048 (0.000-29.505)	0.354	0.000 (0.000-1.254)	0.962
CA19-9 (< 37.0 U/mL *vs* ⩾ 37.0 U/mL)	1.385 (0.883-2.172)	0.156	1.179 (0.710-1.958)	0.525
Overall survival				
Age (< 60 *vs* ⩾ 60 yr)	1.047 (0.816-1.342)	0.719	1.218 (0.905-1.639)	0.193
Sex (male *vs* female)	0.979 (0.755-1.269)	0.874	0.995 (0.732-1.355)	0.977
Lesions (single *vs* multiple)	1.144 (0.804-1.626)	0.455	1.163 (0.769-1.761)	0.474
T staging (T 0-2 *vs* T 3, 4)	2.498 (1.846-3.382)	< 0.001	1.437 (0.986-2.094)	0.059
N staging (N 0, 1 *vs* N 2, 3)	3.577 (2.713-4.715)	< 0.001	2.817 (1.984-4.001)	< 0.001
Differentiation (well *vs* poorly)	1.595 (1.227-2.073)	< 0.001	1.408 (0.990-2.002)	0.057
Histology (adenocarcinoma *vs* signet ring cell cancer)	1.171 (0.880-1.560)	0.279	1.000 (0.702-1.424)	0.999
Lymphovascular invasion (negative *vs* positive)	2.527 (1.739-3.673)	< 0.001	1.004 (0.643-1.569)	0.985
Peritoneal metastasis (negative *vs* positive)	4.620 (3.098-6.6887)	< 0.001	3.213 (1.792-5.762)	< 0.001
Hepatic metastasis (negative *vs* positive)	2.294 (1.285-4.097)	0.005	2.114 (0.916-4.880)	0.079
CA19-9 (< 37.0 U/mL *vs* ⩾ 37.0 U/mL)	1.395 (1.087-1.791)	0.009	1.414 (1.053-1.898)	0.021

CA: Carbohydrate antigen.

Prognostic factors

Potential prognostic variables for DFS are presented in Table 2. In univariate analysis, DFS was significantly associated with T stage (P = 0.001), N stage (P < 0.001), and the presence of lymphovascular invasion (P = 0.002). Other factors, including age, sex, number of lesions, differentiation, histology, peritoneal metastasis, and hepatic metastasis, were not significantly associated with DFS. Only N stage (P = 0.001) remained significantly linked with DFS in multivariate analysis. Neither univariate nor multivariate analyses revealed that preoperative serum CA 19-9 levels affected DFS.

Potential prognostic variables are shown in Table 2. Focusing on OS, univariate analysis demonstrated an association with T stage (P < 0.001), N stage (P < 0.001), differentiation (P < 0.001), the presence of lymphovascular invasion (P < 0.001), the presence of peritoneal metastasis (P < 0.001), and hepatic metastasis (P = 0.005). Multivariate analysis showed that N stage (P < 0.001), and the presence of peritoneal metastasis (P < 0.001) remained independent factors in predicting OS. Additionally, preoperative serum CA 19-9 levels were significantly associated with OS in univariate (P = 0.009) and multivariate (P = 0.021) analyses. Cox proportional hazards regression analysis indicated that patients with elevated levels of CA 19-9 had a 1.4-fold higher risk of worse OS

than patients with low levels of this marker.

DISCUSSION

Gastric cancer is one of the most common cancers worldwide with approximately 989600 new cases and 738000 deaths per year, accounting for approximately 8% of new cancers[25]. Thus, gastric cancer continues to be a global health problem. However, gastric cancer-specific tumor markers have not yet been identified. The tumor markers currently in use have limited clinical utility due to insufficient specificity and poor sensitivity[14,26,27].

Nevertheless, current serum tumor markers are primarily used for the preoperative staging of neoplasms, postoperative monitoring of treatment effectiveness, and early diagnosis of recurrence, as they can be easily and cost-effectively identified[28]. Specifically, tumor markers, including alpha-fetoprotein (AFP), CEA, CA 19-9, CA 50, and CA 72-4, have been reported to be elevated in certain gastric cancer patients[11,19,20]. AFP, a marker commonly used for germ cell and hepatocellular carcinoma, is elevated in AFP-producing gastric cancer, often presenting as liver metastasis and leading to a poor prognosis. However, the value of these tumor markers in gastric cancer is still controversial[29-31]. In the case of CEA, preoperative serum CEA levels have been reported to be useful for determining or predicting gastric cancer prognosis[11,32,33]. However, some authors have indicated that CEA positivity is not a prognostic factor in gastric cancer[15]. In addition, earlier studies have reported that CA 72-4 is more relevant than other tumor markers for gastric cancer, but this has not been verified[13,33-36]. Of currently used markers, CA 19-9 is known to have a positive correlation with depth of invasion, nodal involvement, and peritoneal metastasis in gastric cancer[15,17,37]. However, CA 19-9 can be elevated in endometrial, lung, breast, and pancreatic cancers as well as benign conditions, including cholecystitis, cholangitis, acute pancreatitis, and liver cirrhosis[38,39]. An association between CA 19-9 levels and prognosis has not yet been established and remains controversial[5,13]. Thus, none of these markers are used alone to diagnose, monitor disease, or predict prognosis. Although prognosis is mainly determined by tumor stage at the time of gastric cancer surgery, recent studies have assessed the usefulness of preoperative tumor marker levels to predict invasiveness and prognosis[5,6,18,32,33,40].

Therefore, we focused on the prognostic significance of high preoperative levels of CA 19-9 in patients with gastric cancer. The results of our study indicate that measurements of preoperative serum CA 19-9 levels may be useful in the prediction of survival and prognoses in patients with gastric cancer, confirming its association with OS and DFS. OS is thought to be influenced by preoperative CA 19-9 levels.

Previous studies identified the usefulness of postoperative CA 19-9 levels to predict prognosis and recurrence of gastric cancer after gastrectomy[6,37]. In many studies, however, preoperative CA 19-9 levels were neither prognostic nor were associated with survival. According to studies by Dilege *et al*[17] and Ishigami *et al*[41], preoperative CA 19-9 levels were only significantly correlated with lymph node metastasis; patient survival did not correlate with preoperative CA 19-9 levels. In addition, Ucar *et al*[15] showed that CA 19-9 was only significantly related to lymph node metastasis and peritoneal carcinomatosis, but prognosis and survival were not relevant. These authors suggested that CA 72-4 was an independent prognostic factor for risk of death in this study.

These studies did not provide any predictive information for preoperative CA 19-9 levels on prognosis or survival in gastric cancer patients. One study showed by univariate analysis that preoperative CA 19-9 levels could predict specific clinical outcomes such as DFS. Another study showed poor OS in CA 19-9-positive patients by the log-rank test[5]. However, the independent prognostic value of CA 19-9 on OS by Cox regression multivariate analysis was not shown[5]. The association between CA 19-9 levels and stage of disease, lymph node metastasis, and depth of invasion has been reported, but none have been assigned an independent prognostic value by multivariate analysis[5,16,41]. In contrast, our study showed survival outcome according to preoperative CA 19-9 levels and prognostic factors for OS and DFS. There were significant differences regarding OS between the non-elevated group and the elevated group. Preoperative CA 19-9 levels were a reliable prognostic factor for OS in our study. With respect to DFS, despite an insignificant prognostic value for CA 19-9 levels, we can see possibilities for future research, as our findings are confined to the limited data presented here.

A recent study by Jo *et al*[42] showed that an elevated CA 19-9 concentration before chemotherapy was significantly associated with shorter survival especially in metastatic or recurrent gastric cancer. The patients in this study had metastatic or recurrent gastric cancer. However, our study was designed to analyze treatment-naïve patients who were planning to undergo gastrectomy. Therefore, we have superiority and originality compared to previous studies due to the differences in the subject and focus of study.

We aimed to determine whether the preoperative tumor marker CA 19-9 could provide useful information on clinical outcome and postoperative prognosis similar to other common prognostic factors. Unlike the study by Marrelli *et al*[14], we analyzed not only 432 R0 resection cases, but also 56 palliative gastrectomy cases; of these, 27 cases of bypass surgery were also included. We cannot exclude the possibility that survival rates will appear low due to inclusion of the latter cases and influence tumor progression. These factors might affect the tumor burden and predominance of advanced cancer among marker-positive patients[18].

Our study has limitations associated with its retrospective nature, single-center design and relatively small sample numbers. In addition, preoperative CA 19-9 sampling was not performed at the same time before surgery

due to the retrospective design.

Also, analyses regarding the presence of chemotherapy or radiation therapy after surgery were not performed in this study. Twelve patients who had neoadjuvant chemotherapy were included in this study. However, for patients who had adjuvant chemotherapy or other therapies after surgery, analyses were not performed. Although neither adjuvant nor neoadjuvant chemotherapy showed any clear significant survival benefit in gastric cancer[18], these factors might be crucial in influencing survival, therefore further study is necessary. In the future, it would be interesting to measure CA 19-9 consistently during the preoperative examination before surgery and analyze the effects of additional therapies such as adjuvant chemotherapy or radiation therapy. Therefore, multi-center and prospective studies should be designed to certify the prognostic significance of CA 19-9.

We conclude that OS in gastric cancer patients with elevated CA 19-9 levels was lower than that in patients with non-elevated levels. Serum CA 19-9 can be considered an independent prognostic factor in predicting OS in patients anticipating surgery for gastric cancer.

COMMENTS

Background

Gastric cancer is one of the most common cancers worldwide with approximately 989600 new cases and 738000 deaths per year, accounting for approximately 8% of new cancers. Thus, gastric cancer continues to be a global health problem. However, gastric cancer-specific tumor markers have not yet been identified. The tumor markers currently in use have limited clinical utility due to insufficient specificity and poor sensitivity.

Research frontiers

Recent clinical studies have shown that carcinoembryonic antigen and carbohydrate antigen 19-9 (CA 19-9) are recognized as poor prognostic factors for gastric cancer and are related to its recurrence. The prognostic relevance of such tumor markers in patients with gastric cancer is not comparable with those markers used in other carcinomas. Specifically, CA 19-9 has been reported to be elevated in certain forms of gastric cancer. However, because little research on the prognoses of gastric cancer patients with elevated preoperative CA 19-9 levels has been performed, the clinical significance of preoperative CA 19-9 levels has not been fully verified.

Innovations and breakthroughs

In most current research related to preoperative CA 19-9 levels in gastric cancer, discussions on survival have only focused on overall survival (OS). The association between CA 19-9 levels and stage of disease, lymph node metastasis, and depth of invasion has been reported, but none have been assigned an independent prognostic value by multivariate analysis. This study showed survival outcome according to preoperative CA 19-9 levels and prognostic factors for OS and disease-free survival (DFS). There were significant differences regarding OS between the non-elevated group and the elevated group. Preoperative CA 19-9 levels were a reliable prognostic factor for OS in our study. With respect to DFS, despite an insignificant prognostic value for CA 19-9 levels, we can see possibilities for future research, as the findings are confined to the limited data presented here.

Applications

Prior to surgery, CA 19-9 levels could be used to predict poor prognosis and to suggest adjuvant therapies in early stages of the disease when more aggressive surgical approaches are warranted.

Terminology

Serum CA 19-9 concentrations were measured using a commercial chemiluminescent enzyme immunoassay with a normal upper limit of 37 U/mL. Serum CA 19-9 levels were routinely measured immediately before surgery.

Peer review

The authors have focused on the prognostic significance of high preoperative levels of CA 19-9 in patients with gastric cancer. The results of the study indicate that measurements of preoperative serum CA 19-9 levels may be useful in the prediction of survival and prognoses in patients with gastric cancer, confirming its association with OS and DFS. OS is thought to be influenced by preoperative CA 19-9 levels.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5309

World J Gastroenterol 2013 August 28; 19(32): 5309-5313
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Can trans-anal reinforcing sutures after double stapling in lower anterior resection reduce the need for a temporary diverting ostomy?

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Received: April 10, 2013 Revised: June 16, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

AIM: To evaluate trans-anal reinforcing sutures in low anterior resection using the double-stapled anastomosis technique for primary rectal cancers performed at a single institution.

METHODS: The data of patients who received trans-anal reinforcing sutures were compared with those of patients who did not receive them after low anterior resection. Patients who underwent laparoscopic low anterior resection and the double-stapled anastomosis technique for primary rectal cancer between January 2008 and December 2011 were included in this study. Patients with no anastomosis, a hand-sewn anastomosis, high anterior resection, or preoperative chemoradiation were excluded. The primary outcomes measured were the incidence of postoperative anastomotic complications and placement of a diverting ileostomy.

RESULTS: Among 110 patients, the rate of placement of a diverting ileostomy was significantly lower in the suture group (SG) compared with the non-suture control group (CG) [SG, *n* = 6 (12.8%); CG, *n* = 19 (30.2%), *P* = 0.031]. No significant difference was observed in the rate of anastomotic leakage [SG, *n* = 3 (6.4%); CG, *n* = 5 (7.9%)].

CONCLUSION: Trans-anal reinforcing sutures may reduce the need for diverting ileostomy. A randomized prospective study with a larger population should be performed in the future to demonstrate the efficacy of trans-anal reinforcing sutures.

Key words: Anastomotic leak; Low anterior resection; Rectal neoplasms; Double-stapled anastomotic technique; Reinforcement sutures

Core tip: We have performed trans-anal reinforcing sutures after the double-stapled anastomotic technique to intensify the anastomotic line and to reduce leakage. As a result, we found that the rate of placement of a diverting ileostomy was significantly reduced in cases of performing the trans-anal reinforcing sutures although there was no significant decrease of anastomotic leakage.

Baek SJ, Kim J, Kwak J, Kim SH. Can trans-anal reinforcing sutures after double stapling in lower anterior resection reduce the need for a temporary diverting ostomy? *World J Gastroenterol* 2013; 19(32): 5309-5313 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5309.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5309

INTRODUCTION

Anastomotic leakage is a major problem in patients who undergo rectal cancer surgery. This complication is associated with reoperation, prolonged hospital stay, and high morbidity and mortality. In addition, it can adversely in-

fluence functional and oncologic outcomes[1-4]. An anastomotic leakage rate of 2.5%-12% has been reported[5-8]. Leakage can be the result of a combination of technical, local, and systemic factors. Several risk factors, including old age, male sex, smoking, diabetes, obesity, preoperative chemotherapy, and a more distal tumor location, are associated with anastomotic leakage after rectal cancer surgery[9-12]. In particular, the technical aspects of anastomosis are also very important. Leakage rates have also been used as an indicator of surgical quality[13,14].

Since being introduced by Griffen *et al*[15] and Knight *et al*[16], the double-stapled anastomotic technique has been widely used in colorectal surgery because it allows the anastomosis to be made very low in the pelvis and preserves the anal sphincter[17]. However, this technique creates stapled corners known as "dog ears", which are made by crossing at least two staple lines and become potentially vulnerable areas[18]. The staple line may also be weakened through friction created by hard stools, increasing the risk of anastomotic failure[19].

To address these problems, various methods, such as the single-stapled, double-pursestring method, and bioabsorbable staple-line reinforcement, have been suggested[18,20]. The trans-anal reinforcing suture is another such improvement that has been proposed. We hypothesized that placing the sutures along the staple line, including the corners, can reinforce the anastomosis and reduce anastomotic leakage. Therefore, we are currently using trans-anal reinforcing sutures for low anterior resection. The aim of this study was to determine the effect of trans-anal reinforcing sutures in terms of anastomotic complications and diverting stoma placement.

MATERIALS AND METHODS

Between January 2008 and December 2011, patients who underwent rectal resection at Korea University Anam Hospital for primary rectal cancer were enrolled in this study. The patients who underwent laparoscopic low anterior resection and double stapled anastomosis and had an anastomotic line located within 5-6 cm of the anal verge where trans-anal suturing is possible were included. The exclusion criteria were as follows: intersphincteric resection and coloanal anastomosis, total abdominal colectomy and ileo-rectal anastomosis, abdominoperineal resection, Hartmann's operation, transanal resection and high anterior resection, and a history of receiving chemoradiotherapy preoperatively.

We have been utilizing trans-anal reinforcing sutures since January 2010. A schematic view of the procedure and trans-anal view are shown in Figures 1 and 2. After rectal division using an endo-linear cutter (Echelon, Ethicon), end-to-end anastomosis is performed using a circular stapler (CDH 29 mm, Ethicon), and trans-anal reinforcing sutures are used via the anal canal. Six to eight interrupted sutures are placed along the staple line circumferentially, and two corners made by crossing circular and linear staple lines are always included. An air leakage test is performed for all patients after anastomosis and

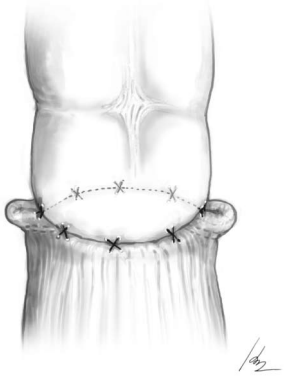

Figure 1 Schematic view of the trans-anal reinforcing sutures. Six to eight interrupted sutures are placed circumferentially along the anastomotic line located within 5-6 cm of the anal verge *via* the anal canal, including the two corners.

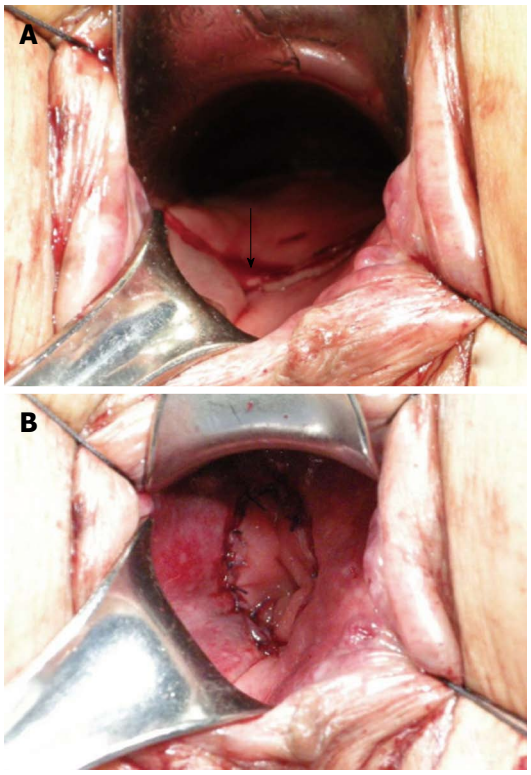

Figure 2 Trans-anal view. A: Crossing point (arrow); B: Reinforcing sutures.

trans-anal reinforcing suture, if done. Temporary diverting ileostomy is considered in cases with several operative or preoperative risk factors such as: a positive air leakage test, insufficient vascular supply at colonic section, several stapling for rectal division, incomplete circular stapling donut, underlying cardiovascular disease, rectal wall muscle injury, and stool spillage. We do not perform ostomy in all male patients.

Clinical anastomotic leakage is defined in the event of clinical symptoms of sepsis, including abdominal pain, tenderness, fever, or leukocytosis. All patients diagnosed with anastomotic leakage in this study were identified within 30 d. Clinical leakage signs were discharge of gas, pus, or feces through the abdominal drain, rectum, or vagina, fecal peritonitis, abscess at the level of the anastomosis, and fluid/air bubbles surrounding the anastomosis on computed tomography (CT). Asymptomatic anastomotic leakages were not considered because routine contrast

Table 1 Patient demographics, tumor characteristics, and operative records

	Suture group (n = 47)	Control group (n = 63)	P value
Sex			0.196
Male	29 (61.7)	31 (49.2)	
Female	18 (38.3)	32 (50.8)	
Age (yr) (range)	64.1 ± 9.8 (39-80)	61.4 ± 11.0 (42-82)	0.199
BMI (kg/m^2) (range)	24.1 ± 3.1 (18.5-33.7)	23.5 ± 2.7 (17.9-28.8)	0.272
Tumor level (cm above AV) (ranges)	9.7 ± 3.9 (2-15)	9.7 ± 3.6 (4-15)	0.974
Diverting ileostomy	6 (12.8)	19 (30.2)	0.031
Length of the operation (min) (ranges)	198.3 ± 75.7 (90-477)	212.1 ± 65.0 (75-335)	0.305
Estimated blood loss (mL) (ranges)	174.5 ± 348.0 (0-2000)	188.4 ± 301.5 (0-1500)	0.823

Data are expressed as absolute numbers (percentage) or mean ± SD. BMI: Body mass index; AV: Anal verge.

Table 2 Postoperative courses

	Suture group (n = 47)	Control group (n = 63)	P value
Flatus (d) (range)	1.5 ± 0.9 (0-4)	1.5 ± 1.2 (0-7)	0.809
Stool (d) (range)	4.1 ± 2.5 (0-10)	3.8 ± 1.7 (1-7)	0.675
Feed (d) (range)	2.8 ± 1.1 (1-6)	2.3 ± 1.8 (1-13)	0.103
Postoperative HS (d), (range)	11.0 ± 5.6 (4-36)	9.8 ± 6.7 (5-44)	0.321
Complications	4 (8.5)	7 (11.1)	0.656
Anastomotic leakage	3 (6.4)	5 (7.9)	0.759
Conservative management	1	3	
Reoperation	2	2	
Intra-abdominal bleeding	0	1 (1.6)	0.390
Postoperative ARF	1 (2.1)	1 (1.6)	0.390

Data are expressed as absolute numbers (percentage) or mean ± SD. HS: Hospital stay; ARF: Acute renal failure.

enemas were not performed after surgery. Patients who developed leakage were treated conservatively with antibiotics, received CT or ultrasonography guided drainage, or were treated with reoperation under general anesthesia.

All data were prospectively collected in a database and analyzed under the approval of the Institutional Review Board. Patient demographics, tumor characteristics, operative records, and postoperative courses were compared between patients who had trans-anal reinforcing sutures and those who did not. Statistical analysis was performed using SPSS version 12.0 (Chicago, IL). Student's t-test was used to compare continuous variables. χ^2 test was used to compare discrete variables. $P < 0.05$ was considered statistically significant.

RESULTS

In total, 110 patients underwent laparoscopic low anterior resection with double-stapled anastomosis for primary rectal cancer [47 in the suture group (SG), and 63 in the non-suture control group (CG)]. Relevant patient characteristics and surgical histories are shown in Table 1. No significant difference was observed in sex, age, or body mass index (BMI) between groups. There was also no difference in mean tumor level (9.7 cm *vs* 9.7 cm from the anal verge, $P = 0.974$), mean length of operation (198.3 min *vs* 212.1 min, $P = 0.305$) or estimated blood loss (174.5 mL *vs* 188.4 mL, $P = 0.823$) between groups. The number of temporary diverting ileostomies performed was significantly higher in the control group [SG, $n = 6$ (12.8%); CG, $n = 19$ (30.2%), $P = 0.031$].

The postoperative courses are outlined in Table 2. No significant differences were observed in the time to postoperative flatus (1.5 d *vs* 1.5 d, $P = 0.809$), stool passage (4.1 d *vs* 3.8 d, $P = 0.675$), feeding (2.8 d *vs* 2.3 d, $P = 0.103$), or postoperative hospital stay (11.0 d *vs* 9.8 d, $P = 0.321$). The incidence of anastomotic leakage, which was not significant between groups ($P = 0.759$), was 6.4% in the SG ($n = 3$) and 7.9% in the CG ($n = 5$). Two patients in each group required reoperation for anastomotic leakage, while others were treated conservatively. There were no differences in other complications between the two groups.

DISCUSSION

The occurrence of anastomotic leakage is a major concern in rectal cancer surgery. The consensus is that the main causes of anastomotic leakage are ischemia and tension. Among the risk factors for anastomotic leakage, the technical aspects of surgery are very important as they are the only known factors that may be corrected. In the double-stapled anastomotic technique, at least two staple lines cross each other, creating vulnerable corners. Some reports have concluded that the anastomotic technique used is not an important factor in anastomotic leakage, however some controversy still exists[21].

Various attempts to modify the technical aspects in order to reduce the problem of the double-stapled anastomotic technique have been attempted. Marecik *et al*[18] used the single-stapled, double pursestring technique for colorectal anastomosis in 160 patients who underwent anterior resection of the upper- or mid-rectum, which resulted in an extremely low rate of anastomotic leakage (0.6%). Mukai *et al*[22] reported good results in two cases in which trans-anal reinforcing sutures after double-stapling for lower rectal cancer were used. Gadiot *et al*[19] compared 76 patients who received anti-traction sutures and 77 who did not, and found that the need for placement of a diverting ostomy was significantly lower in patients who received sutures.

In our study, there was no significant difference in anastomotic leakage between those who received trans-anal reinforcing sutures and those who did not. How-

ever, the need for temporary diverting ileostomy was significantly lower in the suture group, which is the most important outcome in this study. Although some controversy exists as to whether or not proximal diversion affects leak rates[12,23,24], diverting ileostomy may play a role in moderating symptoms or signs of anastomotic leakage to subclinical levels. Consequently, leakage rates may be underestimated in patients who undergo diverting ileostomy. Thus, the actual rate of anastomotic leakage in the control group, which had more ileostomies, was possibly higher than presented.

Meanwhile, trans-anal reinforcing sutures could reduce the need for placement of a diverting ileostomy. It may be due to the decrease in positive air leakage although we cannot present absolute numbers because we believe that the other risk factors for anastomotic leakage were similar between groups. Less air leakage means that trans-anal reinforcing sutures can reduce potential anastomotic leakage by serving as a mechanical safety mechanism. We believe this procedure can be a useful method for the prevention of mechanical failure by reducing anastomotic tension. Therefore, the need for less ileostomy in the suture group is clinically meaningful.

In addition, this procedure can provide emotional stability to surgeons. The placement of stoma usually depends on the surgeon's subjectivity. Apart from the cases where stoma definitely need to be made, many diverting stoma are made due only to the surgeon's insecurity. Although the trans-anal reinforcing sutures may not prevent definite major anastomotic leakage or may not reduce diverting stoma made due to the evident risk, it is believed that this procedure has a positive effect in that it decreases the number of unnecessary stoma by indirectly enhancing surgeons' emotional stability.

While diverting ileostomy is an important procedure for patients at risk for anastomotic leakage, it also carries the potential for many complications and is inconvenient for patients[25-27]. Complications related to ostomy include herniation, retraction, prolapse, stenosis, stoma ischemia, mucocutaneous suture line, and skin problems such as irritant contact dermatitis, inflammatory damage, or allergic reaction. Moreover, systemic complications such as dehydration may occur. In addition, surgery is required at least once more, which can impact patient quality of life and may result in poor cosmesis[28]. Therefore, unnecessary placement of an ileostomy should be avoided. If a simple procedure such as trans-anal reinforcing sutures can reduce the incidence of ileostomies, its use should be considered.

In our results, there was no significant difference between the suture group and the non-suture group in terms of operation time as it takes about 5-0 min to perform the trans-anal reinforcing sutures. Considering that the main disadvantages of using the single-stapled technique include the extra time needed and the potential for pelvic contamination[18], the trans-anal reinforcing suture method is easy and efficacious without additional time or complexity. As this procedure is not different from the one used at the time of trans-anal excision or hemorrhoid surgery, thus it is very familiar to surgeons and a specific learning curve for it may not be necessary even in male patients with narrow pelvises. The only precaution that may need to be taken concerns a risk of vaginal fistula in cases of deep sutures of the female anterior part. This risk should be kept in mind.

Another advantage of trans-anal reinforcing sutures is that anastomotic bleeding can be prevented. Anastomotic bleeding may occur at the staple line and sometimes requires hemostasis with endoscopy or surgery. Thus, routine trans-anal inspection and suturing could aid in the detection of anastomotic bleeding and thereby prevent the increase in rectal pressure due to blood collection.

Our study has several limitations. First, there may have been selection bias in the decision to place a diverting ileostomy since the decision for ileostomy is solely the surgeon's. Our results showed that the incidence of temporary diverting ileostomy was significantly lower in the suture group. Even so, one advantage of this procedure is that it may reduce the number of unnecessary diverting ileostomies made due to the surgeon's excessive anxiety. Second, this study was not randomized, and there was a difference between the two groups when the surgeries were performed. The time difference may be the result of bias due to the surgeon's experience and may have affected the results of the procedures or the postoperative courses. However, the effects of this bias may not be significant since the surgeon performing the procedures in this study was very experienced and had performed a large volume of cases prior to the study period. Third, the sample size was relatively small. Thus, a randomized prospective study should be conducted in a larger population in the future.

In conclusion, our study demonstrates that trans-anal reinforcing sutures can be performed easily and safely in patients undergoing low anterior resection using the double-stapled anastomosis technique for primary rectal cancer. This procedure may reduce the number of diverting ileostomies performed. A prospective randomized trial is necessary to evaluate the effect of trans-anal reinforcing sutures on anastomotic leakage as well as the necessity of the placement of stomas.

COMMENTS

Background

Anastomotic leakage is a major problem in patients who undergo rectal cancer surgery. This complication is associated with reoperation, prolonged hospital stay, and high morbidity and mortality. In addition, it can adversely influence functional and oncologic outcomes. Leakage can be the result of a combination of technical, local, and systemic factors. Several risk factors, including old age, male sex, smoking, diabetes, obesity, preoperative chemotherapy, and a more distal tumor location, are associated with anastomotic leakage after rectal cancer surgery.

Research frontiers

To address these problems, various methods, such as the single-stapled, double-pursestring method, and bioabsorbable staple-line reinforcement, have been suggested. The trans-anal reinforcing suture is another such improvement that has been proposed.

Innovations and breakthroughs

This study was conducted to determine the effect of trans-anal reinforcing su-

tures in terms of anastomotic complications and diverting stoma placement.

Applications

This study demonstrates that trans-anal reinforcing sutures can be performed easily and safely in patients undergoing low anterior resection using the double-stapled anastomosis technique for primary rectal cancer.

Peer review

This paper addresses an important issue which is of interest to most surgeons. Anastomotic breakdown carries a major morbidity and mortality. Any procedure that attempts to reduce this is welcome.

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P- Reviewers Abou-Zeid AA, Amin I, Michot F
S- Editor Zhai HH **L- Editor** O'Neill M **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5314

World J Gastroenterol 2013 August 28; 19(32): 5314-5319
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

HEF-19-induced relaxation of colonic smooth muscles and the underlying mechanisms

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Received: May 27, 2013 Revised: July 1, 2013
Accepted: July 12, 2013
Published online: August 28, 2013

Abstract

AIM: To investigate the relaxant effect of chromane HEF-19 on colonic smooth muscles isolated from rabbits, and the underlying mechanisms.

METHODS: The relaxant effect and action mechanisms of HEF-19 were investigated using descending colon smooth muscle of the rabbits. Preparations 1 cm long were mounted in 15-mL tissue baths containing Tyrode's solution, maintained at 37 ± 0.5 ℃ and aerated with a mixture of 5% CO_2 in oxygen (Carbogen). The tension and amplitude of the smooth muscle strips were recorded after adding HEF-19 (10^{-6}, 10^{-5} and 10^{-4} mol/L). After cumulative administration of four antispasmodic agents, including acetylcholine chloride (Ach) (10^{-4} mol/L), histamine (10^{-4} mol/L), high-K^+ (60 mmol/L) and $BaCl_2$ (8.2 mmol/L), HEF-19 (3×10^{-7}-3×10^{-4} mol/L) was added to investigate the relaxant effect of HEF-19. $CaCl_2$ (10^{-4}-2.5×10^{-3} mol/L) was added cumulatively to the smooth muscle preparations pretreated with and without HEF-19 (1×10^{-6} or 3×10^{-6} mol/L) and verapamil (1×10^{-7} mol/L) to study the mechanisms involved. Finally, phasic contraction was induced with ACh (15×10^{-6} mol/L), and $CaCl_2$ (4×10^{-3} mol/L) was added to the smooth muscle preparations pretreated with and without HEF-19 (3×10^{-6} mol/L or 1×10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) in calcium-free medium to further study the underlying mechanisms.

RESULTS: HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) suppressed spontaneous contraction of rabbit colonic smooth muscles. HEF-19 (3×10^{-7}-3×10^{-4} mol/L) relaxed in a concentration-dependent manner colonic smooth muscle preparations pre-contracted with $BaCl_2$, high-K^+ solution, Ach or histamine with respective EC_{50} values of 5.15 ± 0.05, 5.12 ± 0.08, 5.58 ± 0.16 and 5.25 ± 0.24, thus showing a spasmolytic activity. HEF-19 (1×10^{-6} mol/L and 3×10^{-6} mol/L) shifted the concentration-response curves of $CaCl_2$ to the right and depressed the maximum response to $CaCl_2$. The two components contracted by Ach were attenuated with HEF-19 (3×10^{-6} mol/L or 10^{-5} mol/L) in calcium-free medium.

CONCLUSION: HEF-19 inhibited rabbit colonic smooth muscle contraction, probably through inhibiting opening of voltage-dependent Ca^{2+} channels. HEF-19 reduced inflow and intracellular release of Ca^{2+} ions.

Key words: Colonic smooth muscle; Smooth muscle relaxation; Ca^{2+} channels

Core tip: This is a good descriptive study in which authors found a new L-calcium-antagonist relaxing rabbit colonic smooth muscles and analyzed its possible mechanism. It provides an opportunity to search for a new drug highly selective to the gastrointestinal tract, effectively relieving pain, diarrhea and intestinal discomfort, but without significant adverse effects on irritable bowel syndrome patients.

Wei YY, Sun LL, Fu ST. HEF-19-induced relaxation of colonic smooth muscles and the underlying mechanisms. *World J Gastroenterol* 2013; 19(32): 5314-5319 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5314.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5314

INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent gastrointestinal disease, characterized by a combination of several symptoms including abdominal pain or discomfort, flatulence, and problems related to bowel habits (constipation and/or diarrhea)[1]. Abnormal contraction of intestinal smooth muscle may be importantin producing the main IBS symptoms. thus, modifying the contractility is often the major aim in the treatment of IBS[2,3]. Calcium channel blockers have a good effect on IBS patients with abdominal pain and diarrhea[4].Calcium channel blockers have received increasing attention in the treatment of IBS. 3.4-Dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride (HEF-19) is a compound with a relaxant effect on colonic smooth muscles.

The present study investigated the relaxant effect of HEF-19 on isolated descending colon smooth muscle from rabbits, and the underlying mechanisms (Figure 1).

MATERIALS AND METHODS

Animals

New Zealand rabbits of either sex (2.0-2.5 kg) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Certificate number: SCXK20030011). All care and handling of animals were approved by the Institutional Animal Ethical Committee.

Chemicals and reagents

Normal Tyrode's solution contained: NaCl 136.86 mmol/L, KCl 2.68 mmol/L, $NaHCO_3$ 11.9 mmol/L, $MgCl_2$ 1.05 mmol/L, KH_2PO_4·H_2O 0.41 mmol/L, $CaCl_2$ 1.8 mmol/L, and glucose 5.6 mmol/L. A high-K^+ solution (KCl, 60 mmol/L) was obtained by equimolar replacement of NaCl by KCl in Tyrode's solution[5]. Ca^{2+}-free Tyrode solution was the solution in which $CaCl_2$ was omitted and ethylenediaminetetra-acetic acid (EDTA, 0.1 mmol/L) was added[6]. Ca^{2+}-free high-K^+ solution was the Ca^{2+}-free and high-K^+ Tyrode solution. All chemicals were dissolved in distilled water. All solutions were stored at 4 ℃ and fresh dilutions were made daily.

HEF-19 (> 99.5% purity) was provided by Organic Chemistry Laboratory of Shenyang Pharmaceutical University and dissolved in distilled water. KCl was from Shenyang Chemical Reagent Factory, Shenyang, China, $CaCl_2$ from Tianjin Bodi Chemical Co., Tianjin, China, $BaCl_2$ from Shenyang Xingdong Reagent Factory, Shenyang, China, verapamil injection from Tianjin Heping Pharmaceutical Plant, Tianjin, China, and acetylcholine chloride (Ach) and histamine were from Sigma, United States.

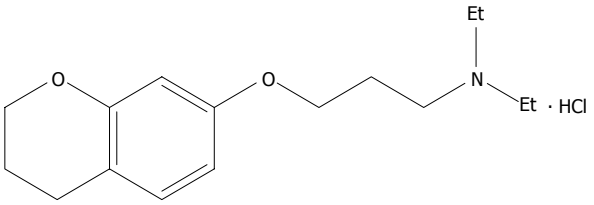

Figure 1 3.4-dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride.

Preparation of colonic smooth muscles

The animals had free access to water but were fasted for 24 h before the experiments. The animals were killed by a blow to the head. The descending colon portion was isolated, washed, and freed from the mesentery. Preparations 1 cm long were mounted in 15-mL tissue baths containing Tyrode's solution maintained at 37 ± 0.5 ℃ and aerated with a mixture of 5% CO_2 in oxygen. A preload of 3 g was applied and the tissues were kept undisturbed for an equilibrium period of 60 min. During that time, the nutrient solution was changed every 20 min. Changes in isometric tension were measured with a force-displacement transducer (Chengdu Instrument Plant, Chengdu, China) and recorded by an RM6240B Multichannel Physiological Signal Collection and Handling System (Chengdu Instrument Plant)[7].

Effect of HEF-19 on spontaneous contraction of rabbit descending colon

The normal tension and amplitude of the descending colonic smooth muscle strips were recorded after the contraction reached a stable plateau. HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) and vehicle were added to the tissue baths containing Tyrode's solution.

Relaxant effect of HEF-19 on contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine

The isolated colon smooth muscle preparations were contracted with Ach (1×10^{-4} mol/L), histamine (1×10^{-4} mol/L) High-K^+ (60 mmol/L) or $BaCl_2$ (8.2 mmol/L), after the contraction reached a stable plateau, and cumulative concentrations of HEF-19 (3×10^{-7} mol/L-3×10^{-4} mol/L) were added. The relaxant effect was expressed as a percentage of relaxation and the EC_{50} (concentration to produce a 50% maximal relaxation) was calculated using a multichannel physiological system.

Inhibition of $CaCl_2$-induced cumulative contractions

The isolated preparations were allowed to stabilize in normal Tyrode's solution and were replaced with Ca^{2+}-free Tyrode's solution for 30 min, and then K^+-rich and Ca^{2+}-free Tyrode's solution. After 15 min incubation, Ca^{2+} was added in a cumulative fashion (1×10^{-4}-2.5×10^{-3} mol/L) to obtain control concentration-response curves. The results were expressed as the percentage of the maximum contractile tension to $CaCl_2$ before and after pretreatment

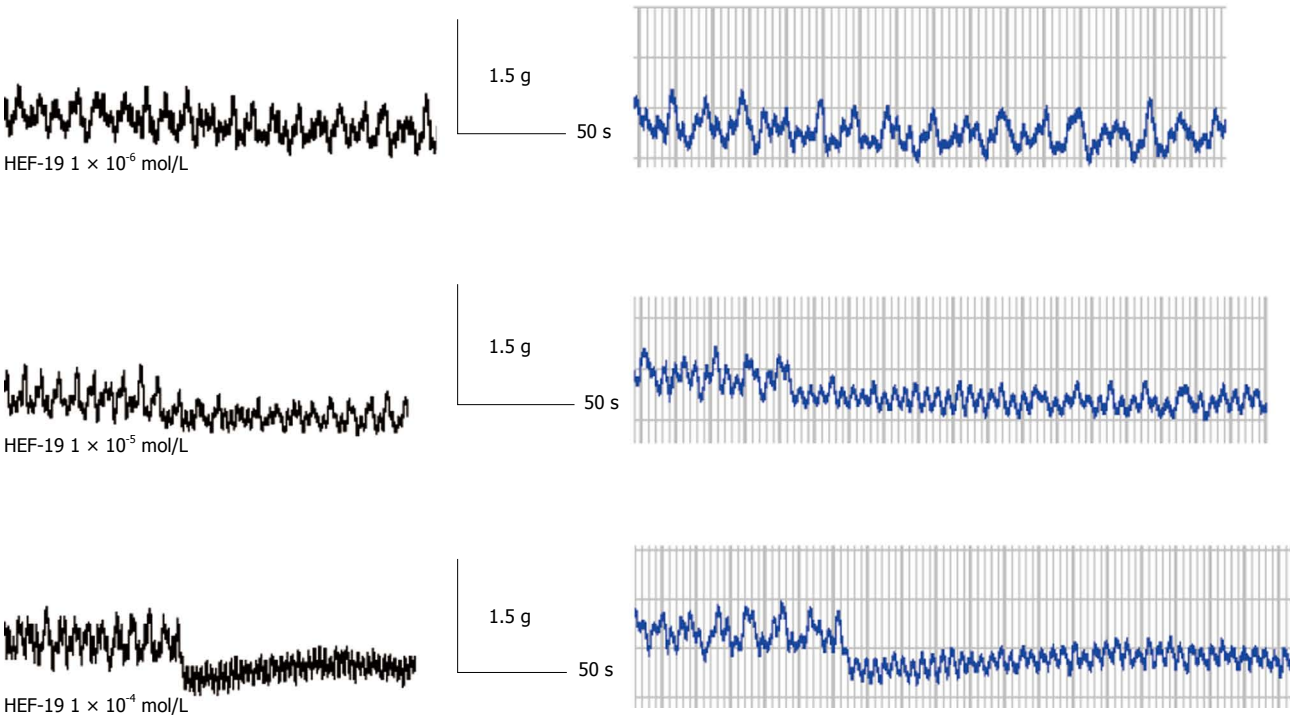

Figure 2 Effects of HEF-19 on spontaneous tension and amplitude of isolated rabbit descending colonic smooth muscle.

Table 1 Effects of HEF-19 on tension and amplitude of spontaneous contraction of descending colonic smooth muscles

Group	After administration (%)	
	Tension	Amplitude
Vehicle	97.98 ± 2.37	103.69 ± 10.13
HEF-19 (mol/L)		
10^{-6}	89.87 ± 2.60	81.96 ± 13.90[a]
10^{-5}	75.98 ± 3.2[b]	48.40 ± 6.07[b]
10^{-4}	55.05 ± 18.13[b]	37.77 ± 2.54[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* vehicle. Values are mean ± SD, $n = 6$.

with HEF-19 (1×10^{-6} or 3×10^{-6} mol/L) and verapamil (1×10^{-7} mol/L) respectively[8].

Inhibition of HEF-19 on biphasic contraction induced by ACh

After the equilibration period, normal Tyrode's solution was replaced with Ca^{2+}-free Tyrode's solution for 20 min. The phasic contraction caused by ACh (15×10^{-6} mol/L) was obtained, and tonic contraction was induced by further addition of $CaCl_2$ (4×10^{-3} mol/L). After washing with normal Tyrode's solution, the experiments were repeated with incubation for 10 min with HEF-19 (3×10^{-6} mol/L or 1×10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) respectively[8,9].

Statistical analysis

Statistical evaluation of the data was performed using Student's *t* test when appropriate. The data were expressed as mean ± SD or mean ± SEM and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of HEF-19 on spontaneous contraction of rabbit descending colon

HEF-19 (1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol/L) significantly suppressed the tension and amplitude of *spontaneous contraction*, in a concentration-dependent manner. Figure 2 is print screen about tension and amplitude of spontaneous contraction of descending colonic smooth muscles. Tension is *y*-axis. Time is *x*-axis. Amplitude is difference between the peaks and troughs. The data of Figure 2 showed in Table 1.

Relaxant effects of HEF-19 in contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine

The maximum responses of the cumulative concentration-response curves to $BaCl_2$, high-K^+ solution, Ach or histamine were depressed by HEF-19 in a dose-dependent manner (3×10^{-7}-3×10^{-4} mol/L). EC_{50} values were 5.15 ± 0.05, 5.12 ± 0.08, 5.58 ± 0.16 and 5.25 ± 0.24 (Figure 3).

Inhibition of $CaCl_2$-induced contraction

The maximum cumulative concentration-response curves for $CaCl_2$-induced contraction were depressed by HEF-19 (1×10^{-6} and 3×10^{-6} mol/L) in a concentration-dependent manner. These results indicated that HEF-19 showed non-competitive antagonism (Figure 4).

Inhibitory effect of HEF-19 on biphasic contraction induced by ACh

The phasic and tonic contraction induced by ACh was decreased by HEF-19 (3×10^{-6} and 1×10^{-5} mol/L) in a

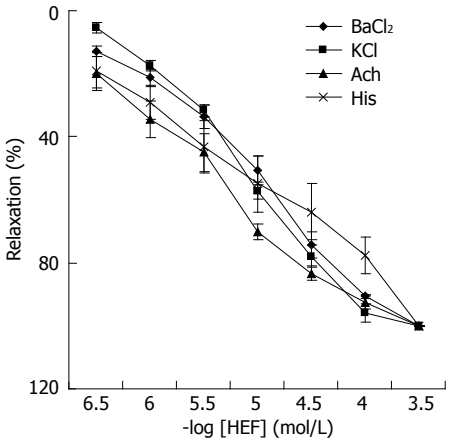

Figure 3 Relaxant effect of HEF-19 (3×10^{-7}-3×10^{-4} mol/L) on isolated rabbit descending colonic smooth muscle pre-contracted with Ach, histamine, high-K^+ solution or $BaCl_2$. Data are mean ± SE (n = 6).

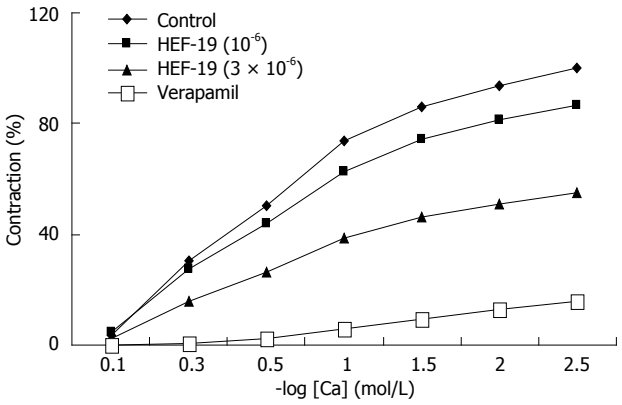

Figure 4 Effect of HEF-19 and verapamil on the contraction-response curve of $CaCl_2$ in descending colonic smooth muscle isolated from rabbits. Data are mean ± SE (n = 6) and are expressed as percentage of maximum contraction.

concentration-dependent manner after pretreatment in calcium-free medium with EGTA (Figure 5).

DISCUSSION

Excitation-contraction coupling in smooth muscle occurs through two main mechanisms. Many smooth muscles are activated by Ca^{2+} signaling cascades. In addition, there is a Rho/Rho kinase signaling pathway that acts by altering the Ca^{2+} sensitivity of the contractile system[10,11]. The predominant source of activator and intracellular Ca^{2+} has little role to play in mediating excitation-contraction coupling by agonists. Both tonic and phasic (rhythmic) contraction are regulated by intracellular Ca^{2+} concentration. Ca^{2+} originates from the intracellular Ca^{2+} store, the sarcoplasmic reticulum, and influx from the extracellular space. Phasic contraction is influenced by neurotransmitters, hormones, and drugs. In circular muscle, these agents can also increase calcium by releasing it from intracellular stores, thus inducing tonic contraction[12-19].

Smooth muscle has the automatic rhythmicity. Spontaneous contraction shows the basic rhythmic depolarization wave. HEF-19 suppressed the spontaneous contractile amplitude and tension of rabbit colonic smooth muscle in a concentration-dependent manner. It has been reported that extracellular Ca^{2+} participates in spontaneous activity and enters the cytosol by L-type voltage-dependent Ca^{2+} channels[20].

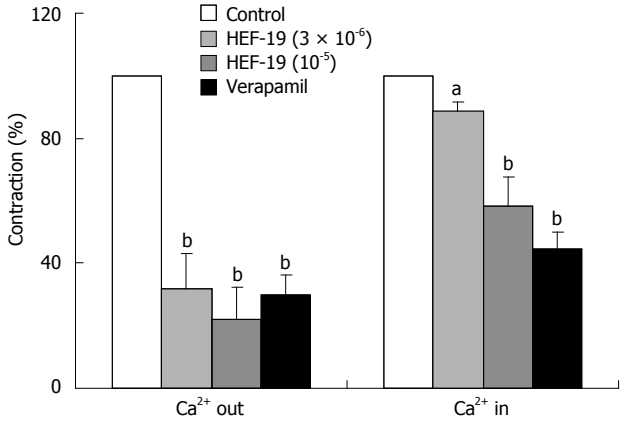

Figure 5 Effects of HEF-19 (3×10^{-6} and 10^{-5} mol/L) and verapamil (1×10^{-7} mol/L) on biphasic contraction induced by Ach in descending colonic smooth muscle isolated from rabbits. Data are mean ± SE (n = 6). $^aP < 0.05$, $^bP < 0.01$ *vs* the controls.

The contraction induced by $BaCl_2$, high-K^+ solution, Ach or histamine was relaxed by HEF-19. High-K^+ elicits an increase in intracellular Ca^{2+} and transient contractions[21,22]. ACh induces smooth muscle contraction via activating muscarinic receptors. Extracellular and intracellular Ca^{2+} participate in the Ach-induced contraction[23]. Histamine has a spasmogenic effect on the gastrointestinal tract through activating histaminergic receptors and increasing Ca^{2+} influx[24,25]. $BaCl_2$ causes cell membrane depolarization and intracellular Ca^{2+} release, and it can cross the cell membrane through the Ca^{2+} channels to bind with troponin directly[26].

HEF-19 depressed the maximum cumulative concentration-response curve for $CaCl_2$ in a non-competitive manner, similar to verapamil. The fact that HEF-19 inhibited $CaCl_2$-induced smooth muscle contraction indicated that it inhibited the voltage-dependent Ca^{2+} channels, because $CaCl_2$ can open these channels during high-K^+ depolarization[27,28].

There are biphasic responses, including fast and slow components, in the contraction induced by ACh. The fast (phasic) phase is due to the release of intracellular Ca^{2+} induced by ACh in Ca^{2+}-free medium[21], and the sustained (tonic) phase is largely dependent on the influx of external Ca^{2+} resulting from the reintroduction of $CaCl_2$ into the medium. HEF-19 decreased the phasic and tonic contraction. The results showed that HEF-19 eventually inhibited the Ca^{2+} channels to reduced release of intracellular Ca^{2+} and influx of external Ca^{2+}.

In conclusion, our results suggest that HEF-19 relaxed rabbit descending colonic smooth muscle by blocking voltage-dependent Ca^{2+} channels. HEF-19 inhibited

the inflow of extracellular Ca^{2+} into cells, and intracellular release of Ca^{2+} ions. Ca^{2+} channels blocking effect of HEF-19 is fewer than verapamil on colonic smooth muscle. Calcium channel blockers are also reported to be effective in the treatment of IBS[3]. However, the adverse effects on the cardiovascular system of these blockers limit their further application on IBS patients. HEF-19, a L-type calcium channel blocker with selectivity for the gastrointestinal tract, is expected to be a safe and effective drug for treatment of abdominal pain and diarrhea symptoms associated with IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder in which abdominal pain is associated with changes in bowel habits and abdominal distension. Abnormal contraction of intestinal smooth muscle may be important in producing the main symptoms of IBS, thus, modifying contractility is often the major aim of treatment. Traditional cholinolytic and opioid drugs have been reported to have much adverse reactions. Some enteric spasmolytics agents have been found to treat IBS by selectively blocking voltage-dependent Ca^{2+} channels.

Research frontiers

Current IBS pathophysiologic mechanisms are based on the abnormalities of brain-gut axis. With in-depth researches on various neurotransmitters, ion channel and receptors, designed as targets, new drugs are expected to appear against IBS. Since Pinaverium Bromide was developed and used clinically, there has been increasing concern to search for highly selective blockers of voltage-dependent Ca^{2+} channels to treat IBS patients with abdominal pain and diarrhea.

Innovations and breakthroughs

Chromane HEF-19 has a relaxant effect on colonic smooth muscles. It has previously been shown to have little activity on isolated vascular smooth muscle. The present study investigated the relaxant effect of HEF-19 on isolated descending colon smooth muscle of rabbits and the possible mechanisms. HEF-19 is expected to be a highly selective enteric spasmolytics agent through inhibition of opening of voltage-dependent Ca^{2+} channels in colonic smooth muscle. This is a potentially interesting study to find a drug for treatment of abdominal pain and diarrhea associated with IBS.

Applications

HEF-19 is expected to be a safe, effective and economic drug for treatment of abdominal pain and diarrhea symptoms associated with IBS.

Terminology

HEF-19: HEF-19, 3.4-dihydro-7-[3-(diethylamino) propoxy] chroman hydrochloride, is highly selective enteric spasmolytics agent. IBS is a functional gastrointestinal disorder in which abdominal pain is associated with changes in bowel habits and abdominal distension. People with a functional gastrointestinal (GI) disorder have frequent symptoms, but the GI tract is not damaged. IBS is a group of symptoms that occur together. The most common symptoms of IBS are abdominal pain or discomfort, often reported as cramping, along with diarrhea, constipation, or both.

Peer review

Very well written manuscript. In the manuscript entitled "HEF-19-induced relaxation of colonic smooth muscles and the underlying mechanisms", the authors investigated the relaxant effect of chromane HEF-19 on colonic smooth muscles isolated from rabbits. This is a good descriptive study on a hot topic. The research is well done. The result is well discussed.

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P- Reviewers Chorny M, Suzuki A, Wenzel SE
S- Editor Wang JL **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5320

World J Gastroenterol 2013 August 28; 19(32): 5320-5325
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Prevalence of hepatitis C infection among intravenous drug users in Shanghai

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Author contributions: Tao YL and Tang YF carried out the screening tests for antibodies to hepatitis C virus, Recombinant immunoblot assay, and Qualitative tests for hepatitis C virus RNA; Qiu JP, Cai XF, Shen XT and Wang YX participated in the screening tests for antibodies to hepatitis C virus and sample assembly; Zhao XT designed this study, performed the statistical analysis and wrote the paper; all authors read and approved the final manuscript.
Supported by Science and Technology Commission Xuhui District and Xuhui Health Bureau of Shanghai, No. SHXH201226
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Received: April 24, 2013 Revised: July 15, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

AIM: To characterize the prevalence of hepatitis C virus (HCV) infection among Chinese intravenous drug users (IDUs).

METHODS: A total of 432 adult IDUs (95 women and 337 men) in Shanghai were included in the study. The third-generation Elecsys Anti-HCV assay (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305, Mannheim, Germany) was used to screen for antibodies against HCV. The RIBA strip, a supplemental anti-HCV test with high specificity, was performed on all of the samples that tested positive during the initial screening. All of the anti-HCV positive samples were analyzed with a Cobas TaqMan 48 Analyzer (Roche Diagnostics) for direct detection of HCV RNA. All of the HCV RNA-positive samples were sequenced for genotype determination.

RESULTS: The preliminary screening identified 262 (60.6%) subjects who were seropositive for HCV. Of the 62 females and 200 males seropositive subjects, 16 (16.7%) and 65 (19.3%), respectively, were confirmed by RIBA, yielding an overall HCV seropositive rate of 18.8%. Four female (6.5%) and 14 male (7.0%) subjects tested positive for HCV RNA, indicating an active infection rate of 4.2% for the entire study population. The 18 HCV RNA-positive serum samples were genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two with genotypes 1a and 2a, and two with genotypes 1b and 2a. The active infection rate among HCV-seropositive individuals was 22.2%, which was significantly lower than most estimates.

CONCLUSION: The prevalence of HCV is relatively low among IDUs in Shanghai, with a spontaneous recovery rate much higher than previous estimates.

Key words: Hepatitis C; Anti-hepatitis C virus antibodies; Prevalence of hepatitis C virus; Active infection rate; Intravenous drug users

Core tip: In this report, we examined the prevalence of anti-hepatitis C virus (HCV) antibodies, as well as chronic viremia, in 432 intravenous drug users (IDUs) in Shanghai, China. Our data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

Tao YL, Tang YF, Qiu JP, Cai XF, Shen XT, Wang YX, Zhao XT. Prevalence of hepatitis C infection among intravenous drug users in Shanghai. *World J Gastroenterol* 2013; 19(32): 5320-5325

Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5320.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5320

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus with a diameter of approximately 50 nm, and it is classified as a *Hepacivirus* within the *Flaviviridae* family[1]. Humans are the primary reservoir of HCV; however, the virus has been transmitted experimentally to chimpanzees[2]. The HCV genome consists of a 9.6-kb single-stranded, positive-sense RNA molecule containing one long open reading frame (ORF). This single ORF encodes a large (approximately 3000 amino acids) polyprotein that undergoes co- and post-translational cleavage by host and viral proteases to yield individual viral proteins[1,2]. The N-terminal quarter of the genome encodes core and structural proteins; these proteins consist of a non-glycosylated nucleic acid-binding nucleocapsid protein (core) of 190 amino acids (approximately 21 kDa) and two membrane-associated glycoproteins (E1 and E2/NS1) of 190 and 370 amino acids, respectively (33 and 70 kDa, respectively, when glycosylated). The remaining three-quarters of the genome encode nonstructural proteins NS2-NS5. The NS2 (250 amino acids), NS3 (500 amino acids) and NS4A proteins interact to mediate processing of the presumed NS region of the polyprotein. NS3 is both a proteolytic cleavage enzyme and a helicase, which facilitates unwinding of the viral genome during replication. NS5b is the RNA-dependent RNA polymerase necessary for viral replication[3-5].

Seven HCV genotypes with several distinct subtypes have been identified worldwide[2]. HCV is the etiological agent of hepatitis C. HCV infections are often asymptomatic; however, chronic infection can result in the scarring of the liver, which can ultimately lead to cirrhosis[6-8]. Carriers who develop cirrhosis are at significantly greater risk for developing liver failure, liver cancer or life-threatening esophageal and gastric varices[8]. No effective anti-HCV vaccines are currently available[9,10]. The standard of care therapy for patients with HCV infection is the use of both peginterferon and ribavirin. These drugs are administered for either 48 wk (HCV genotypes 1, 4, 5 and 6) or 24 wk (HCV genotypes 2 and 3). These therapies induce a sustained virologic response (SVR) in infected individuals. SVR rates of 40%-50% are observed in patients with genotype 1 infections, and rates of > 80% are observed in those with genotype 2 and 3 infections[8]. Once achieved, SVRs are associated with the long-term clearance of HCV infection, as well as improved morbidity and mortality[8].

Two major advances have occurred in recent years: the development of direct-acting antiviral (DAA) agents; and the identification of several single-nucleotide polymorphisms associated with spontaneous and treatment-induced clearance of HCV infection[11-19]. Although peginterferon and ribavirin remain vital components of therapy, the emergence of DAAs has led to a substantial improvement in SVR rates, along with the option of abbreviated therapy for many patients with genotype 1 chronic HCV infections[8].

The World Health Organization (WHO) estimates that approximately 3% of the global population has been infected with HCV, including more than 170 million chronic carriers at risk of developing liver cirrhosis and/or liver cancer[2]. HCV transmission occurs primarily through exposure to infected blood[1-9]. Specific routes of infection include intravenous drug use, blood transfusions (before 1992), solid organ transplantation from an infected donor, unsafe medical practices, occupational exposure to infected blood, maternal-fetal transmission, sex with an infected person, high-risk sexual practices and possibly intranasal cocaine use[2]. In China, a nationwide HCV serological survey indicated the prevalence of anti-HCV antibodies to be > 0.5% among more than 80000 Chinese subjects. Furthermore, the rates of hepatitis C were much lower than the rates of hepatitis B among clinical inpatient and outpatient populations[20]. Beginning in the early 1990s, the strict screening of blood donors and precise control over the blood supply were implemented by the Chinese government, which effectively eliminated the transmission of many infectious diseases due to blood transfusions. The majority of HCV infections are now limited to specific subpopulations, such as intravenous drug users (IDUs) and patients with certain hemopathies.

Although the prevalence of HCV is greater among IDUs than in the general population, the infection rates of HCV and other diseases remain unknown among IDUs in China. Many hepatologists and virologists worldwide believe that as high as 40%-80% of individuals infected with HCV will develop chronic hepatitis C[2,8]; however, the true rate at which patients develop chronic hepatitis C remains is not known. This gap in understanding regarding the natural course of HCV infection could lead us to misjudge the true burden of HCV infection and might negatively impact clinical decision-making.

In this report, we examined the prevalence of anti-HCV antibodies, as well as chronic viremia, in 432 IDUs in Shanghai, China. Our data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

MATERIALS AND METHODS

Study population

There are 17 districts in Shanghai, and each district contains one medical center that was established by the local government, where IDUs can receive diaminon therapy for heroin addiction on a regular basis. The total population of Shanghai is approximately 16 million, and Xuhui District is one of the central districts. The residential population of Xuhui District is approximately 1.2 million.

Our samples were collected from Xuhui District. There are approximately 500 IDUs in this district annually, who are treated at the medical center in Xuhui District, where they receive diaminon therapy. A total of 432 adult IDUs, primarily heroin users, were included in this study. Patient serum was collected every 6 mo to monitor HCV, HIV and *Treponema pallidum* (*T. pallidum*) subspecies *pallidum* infections. The participants reported no malaise, weakness, anorexia, jaundice or other symptoms of hepatitis, and they had not previously been diagnosed with viral hepatitis. Accordingly, all of the participants were negative for prior HCV therapy. All of the serum samples used in this study were collected in 2012. Written informed consent was obtained according to the guidelines of the National Ethics Regulation Committee, and the study was approved by the Internal Review Board of the Center for Disease Control and Prevention of Shanghai. The participants were informed of their right to withdraw consent. Consent could be withdrawn by participants, immediate relatives, caregivers or legal guardians.

Screening tests for antibodies to HCV

A third-generation Elecsys Anti-HCV assay (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305, Mannheim, Germany) was used to screen for antibodies against HCV, according to the manufacturer's instructions. The assays were performed using a Cobas 411 e-analyzer. The cutoff index values used for determination of positive reactivity were set based upon the manufacturer's recommendation. Samples with a cutoff-index < 0.9 were considered non-reactive in the Elecsys Anti-HCV assay. Samples having a cutoff-index between $\geqslant 0.9$ and < 1.0 were considered borderline, whereas samples with a cutoff-index of $\geqslant 1.0$ were considered reactive.

Recombinant immunoblot assay

The recombinant immunoblot assay (RIBA) strip, a supplemental anti-HCV test with high specificity, was performed on all of the samples that tested positive during the initial screening. The assays were performed using an MP Diagnostics HCV BLOT 3.0 (MP Biomedicals, Solon, OH, United States), according to the manufacturer's instructions.

Qualitative tests for HCV RNA

A Cobas AmpliPrep Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) was used to isolate HCV RNA from serum samples that tested positive for anti-HCV antibodies during the initial screening. Isolation was performed in accordance with the manufacturer's instructions. All of the samples were then analyzed using a Cobas TaqMan 48 analyzer (Roche Diagnostics) for direct detection of HCV RNA.

HCV genotyping

HCV RNA was extracted from 200 μL of EDTA-treated plasma for each HCV RNA-positive sample, using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. All of the primers were designed on the basis of consensus sequences, as reported by Duarte *et al*[21]. Two sets of primers were designed: one for the 5'-UTR region (for genotypes 1-6), and the other for the NS5B region (supplemental primers for genotypes 1a and 1b). Reverse transcription reactions were conducted with a Reverse Transcription Kit (Biovisualab, Shanghai, China). Multiplex PCR was then performed using a HiFiFast PCR high-fidelity DNA polymerase mix (Biovisualab). PCR was conducted in a Peltier Thermal Cycler (MJ96+/MJ966) under the following conditions: incubation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 10 s; annealing at 58 ℃ for 30 s; and extension at 72 ℃ for 20 s. There was then a final extension step at 72 ℃ for 3 min, and the reactions were held at 4 ℃ thereafter. For genotype determination, direct sequencing was performed bidirectionally using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, United States) using 10 ng of QIAquick Spin-purified PCR product (Qiagen) and either the sense or antisense PCR primer, followed by detection on an ABI 310 automated sequencer (PE Applied Biosystems).

Statistical analysis

The results are expressed as the mean ± SD. The statistical analyses were performed using either Student's *t*-test or analysis of variance with *post hoc* Scheffe correction when appropriate. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Overview of study participants

A total of 432 adult IDUs, ranging from 23 to 63 years of age (mean age 44 ± 9 years old), were enrolled in the study. Of the study participants, 337 were male, and 95 were female. The average history of heroin use was 15 ± 5 years (ranging from 2 to 40 years). The majority of participants administered heroin by injection; all denied sharing syringes. All of the participants were seen at a medical center in Shanghai, where they receive diaminon therapy for heroin addiction on a regular basis. Blood samples were collected every 6 mo to screen for HCV, HIV and *T. pallidum* infections. The participants reported no malaise, weakness, anorexia, jaundice or other symptoms of hepatitis, and they had not previously been diagnosed with viral hepatitis. Accordingly, all of the participants were negative for prior HCV therapy.

Prevalence of antibodies against HCV

According to recommendations put forth by the United States Centers for Disease Control and Prevention (CDC), the detection of anti-HCV antibodies requires the use of a screening test with high sensitivity. In addition, reactions with low positivity should be verified by RIBA or

PCR to confirm the presence of viral RNA[22,23].

Preliminary screening tests for HCV were performed using Elecsys assays and a Cobas 411 e-analyzer. Of the 95 females subjects tested, 65.3% were positive for antibodies against HCV. Of the 337 males subjects, 59.3% were positive for antibodies against HCV. No significant differences in infection rates were observed between the men and women. The overall prevalence of anti-HCV antibodies was 60.6%. These results demonstrate a rate of HCV infection among IDUs that is substantially higher than that in the general population.

The sensitivity of the anti-HCV assay was significantly greater than that of the clinical measurements. For the 262 HCV-seropositive individuals, the cutoff index values ranged from 1.6 to 20.1, with an average of 5.7 ± 3.7, well above the standard 1.0 cutoff index value for positive reactivity.

HCV seropositivity and active infection rates confirmed by RIBA and PCR

To confirm the presence of viral RNA, we reanalyzed the 262 HCV-seropositive subjects using RIBA and PCR. Of the 62 females and 200 males subjects, 16 (16.7%) and 65 (19.3%) were confirmed to be true positives for anti-HCV antibodies, respectively. Therefore, the true HCV-positive rate of our study subjects was 18.8%. All of the RIBA-positive subjects were seropositive for core proteins. Eight subjects displayed a weak or no reaction to NS3-1, whereas 16 failed to display strong reactivity to NS3-2. Roughly half of the 81 subjects were positive for antibodies against NS4 and NS5.

To determine the current HCV infection rate, the sera from all 262 seropositive individuals were analyzed using the Cobas AmpliPrep/Cobas TaqMan HCV Test. Of the 62 females and 200 males subjects, 4 (6.5%) and 14 (7.0%), respectively, were positive for HCV RNA, indicating an active infection rate of 4.2% for the entire study population.

The 18 HCV RNA-positive sera were then genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two subjects with genotypes 1a and 2a, and two subjects with genotypes 1b and 2a.

HCV infection rates among HCV-seropositive subjects

HCV remains difficult to both treat and detect due to the high rate of mutation, which severely limits the efficacy of potential vaccines[2]. Current estimates suggest that as many as 70%-90% of infected individuals fail to clear the virus during the acute phase of the disease and therefore become chronic carriers[2,8]. However, the true rate of viral clearance is not known because neither the rate of HCV infection nor the rate of recovery has been established. Some insight into these questions can be drawn from our cohort of IDUs. Of the 16 females and 65 males subjects who tested positive for HCV antibodies, four and 14 subjects were also positive for HCV RNA, respectively (Table 1), yielding an overall clearance rate of 77.8%, which is substantially higher than most estimates[2,8,24,25].

Table 1 Hepatitis C virus current infection rates in anti-hepatitis C virus positive population

	NAT(+)	RIBA(+)	NAT(+)/RIBA(+) (%)
Female	4	16	25.00%
Male	14	65	21.50%
Total	18	81	22.20%

Hepatitis C virus (HCV) current infection rates in anti-HCV positive population were calculated as [number of individuals with nucleic acid testing (NAT)]/total studied population with anti-HCV positive. RIBA: Recombinant immunoblot assay.

DISCUSSION

Since HCV was identified in 1989[26], infection by means of blood transfusion has been virtually eliminated worldwide, limiting the spread of HCV to select populations, particularly IDUs[2]. In China, the prevalence of HCV in the general population is relatively low[20]. However, the number of IDUs is increasing, with the spread of numerous infectious diseases, including viral hepatitis, HIV and *T. pallidum*, subsequently increasing as well. In this report, 18.8% (81/432) of individuals were confirmed to be seropositive for HCV by RIBA testing. Among these individuals, 14 were also positive for HCV RNA, indicating an active infection rate of 4.2% for our cohort. In 1997, the WHO estimated that 3% of the world's population was infected with HCV[2]; however, a recent nationwide survey in China reported an HCV seropositive rate of < 0.5% among more than 80000 Chinese subjects[20], casting doubt on the WHO estimates. The active infection rate of 4.2% observed in our cohort of IDUs is low compared to other reports; however, it is markedly higher than in the general population. These results highlight the importance of studying at-risk populations, including IDUs.

The active infection rate among HCV-seropositive individuals was 22.2% in this study, which is significantly lower than most estimates[2,8,24,25]. Current estimates suggest that as many as 40%-80% of HCV infections will develop into chronic infections[2,8,24,25]. While these estimates are likely inaccurate, studying infection rates among high-risk populations remains difficult. In addition, the susceptibility and specificity of older detection methods, including anti-HCV and viral RNA tests, are low. The data presented here directly challenge assumptions regarding the rate of chronic infection. Our data indicate that as many as 77.8% of individuals were able to clear HCV infections without the need for anti-viral therapy.

A total of 262 (60.6%) subjects tested positive for anti-HCV antibodies during the initial screening stage, of whom only 81 (18.8%) were confirmed by RIBA, indicating that as many as 181 individuals were false positives detected by the Elecsys and Cobas e-analyzers. The ac-

curacy of our findings was supported by use of these automated systems, which remove biases caused by human error.

Despite improvements in technology, false-positive results for anti-HCV antibodies are a well-known problem. A number of conditions have been shown to induce false positives, including high gamma globulin levels, nephritic syndrome, liver diseases, autoimmune diseases, viral or parasitic infections and pregnancy[27,28]. The United States CDC estimates that for immunocompetent individuals, approximately 35% of the anti-HCV enzyme linked immunosorbent assay immunoassay results are false positives. Adjustments to cutoff indices have been insufficient to overcome these issues[27,28], highlighting the need for more accurate screening methods. Although the third-generation Elecsys Anti-HCV assay and RIBA test detect similar antigens, the RIBA test is capable of distinguishing among the antibodies against core, NS3-1, NS3-2, NS4 and NS5 proteins, and this method was used to confirm the Elecsys results.

Anti-HCV antibodies develop during acute infection, generally between 2 and 8 wk after evidence of liver injury[2,8]. Anti-HCV antibodies are generally not detectable in patients with initial signs or symptoms of hepatitis C, with some individuals not testing positive until 6-9 mo after the onset of illness[2,8]. In contrast, hepatitis C viremia can be detected by reverse transcription polymerase chain reaction within a few days after infection[2,8]. In this study, all of the HCV RNA-positive individuals were confirmed to be seropositive for HCV by RIBA, indicating that the rate of early infection was low.

The 18 HCV RNA-positive sera were genotyped. Seven individuals were genotype 1b, and four were genotype 1a. One individual each was infected with genotypes 2a, 2b and 3a. Four subjects were co-infected with multiple strains: two with genotypes 1a and 2a, and two with genotypes 1b and 2a. These data indicate that the genotype distribution in the population is complex.

The diagnosis of hepatitis is made by biochemical assessment of liver function. Initial laboratory evaluations include total and direct bilirubin, alanine aminotransaminase, aspartate aminotransferase, alkaline phosphatase, prothrombin time, total protein, albumin, globulin, complete blood count and coagulation studies[2,8]. In this study, we did not perform the above clinical evaluations. Further investigation and follow-up of affected individuals are ongoing.

ACKNOWLEDGMENTS

The authors thank all of the individuals who supported this research.

COMMENTS

Background

Since the discovery of hepatitis C virus (HCV) in 1989, strict screening measures have virtually eliminated viral transmission through blood transfusions, limiting the spread of HCV to select populations, particularly intravenous drug users (IDUs). The prevalence of HCV infection is relatively low among the general population in China. However, infection rates among high risk populations in China are unknown.

Research frontiers

Many hepatologists and virologists worldwide believe that as high as 40%-80% of individuals infected with HCV will develop chronic hepatitis C; however, the true rate at which patients develop chronic hepatitis C remains is not known. The gap in understanding regarding the natural course of HCV infection could lead us to misjudge the true burden of HCV infection and might negatively impact clinical decision-making.

Innovations and breakthroughs

In this report, the authors examined the prevalence of anti-HCV antibodies, as well as chronic viremia, in 432 IDUs in Shanghai, China. The active infection rate among HCV-seropositive individuals was 22.2%, which was significantly lower than most estimates.

Applications

The data will facilitate the characterization of the prevalence of HCV infection among Chinese IDUs and will complement our understanding of the natural course of HCV infections.

Terminology

The prevalence of anti-HCV antibodies indicates the prevalence of total antibodies against HCV. False positive results for anti-HCV antibodies are a well-known problem. The recombinant immunoblot assay test is capable of distinguishing between antibodies against core, NS3-1, NS3-2, NS4, and NS5 proteins, whereas this method was used to confirm Elecsys results. The active infection of HCV indicates that the HCV RNA could be detected in individual serum by reverse transcription polymerase chain reaction.

Peer review

The paper is well written and gives important epidemiology information of the HCV infection.

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P- Reviewers Cheung TT, McQuillan GM, Venegas M, Yoshida S
S- Editor Wen LL **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5326

World J Gastroenterol 2013 August 28; 19(32): 5326-5333
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Effects of Fufang Biejia Ruangan Pills on hepatic fibrosis *in vivo* and *in vitro*

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Supported by The Major Project of Applied Basic Research Plan of the Scientific and Technological Department of Tianjin, China, No. 06YFJZJC 02900
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Received: May 14, 2013 Revised: July 2, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

AIM: To explore the protective effect and the relevant mechanisms of Fufang Biejia Ruangan Pills (FFBJRGP) on hepatic fibrosis *in vivo* and *in vitro*.

METHODS: Hepatic fibrosis was induced by carbon tetrachloride composite factors. Adult Wistar rats were randomly divided into four groups: normal control group; hepatic fibrosis model group; FFBJRGP-treated group at a daily dose of 0.55 g/kg; and colchicine-treated group at a daily dose of 0.1 g/kg. The effects of FFBJRGP on liver function, serum levels of hyaluronic acid (HA), type Ⅳ collagen (CⅣ), type Ⅲ procollagen (PC Ⅲ), laminin (LN), histopathology, and expression of transforming growth factor (TGF-β1) and Smad3 in hepatic fibrosis were evaluated *in vivo*. The effects of FFBJRGP on survival rate, hydroxyproline content and cell cycle distribution were further detected *in vitro*.

RESULTS: Compared with the hepatic fibrosis model group, rats treated with FFBJRGP showed a reduction in hepatic collagen deposition and improvement in hepatic lesions. Compared with those of the model group, the activities of alanine aminotransferase (62.0 ± 23.7 U/L) and aspartate aminotransferase (98.8 ± 40.0 U/L) in the FFBJRGP-treated group were decreased (50.02 ± 3.7 U/L and 57.2 ± 30.0 U/L, respectively, $P < 0.01$). Compared with those in the model group, the levels of PCⅢ (35.73 ± 17.90 μg/mL), HA (563.82 ± 335.54 ng/mL), LN (89.57 ± 7.59 ng/mL) and CⅣ (29.20 ± 6.17 ng/mL) were decreased to 30.18 ± 9.41, 456.18 ± 410.83, 85.46 ± 7.51 and 28.02 ± 9.45 ng/mL, respectively. Reverse-transcriptase polymerase chain reaction and Western blotting also revealed that expression of TGF-β1 and Smad3 were down-regulated *in vivo*. Cell proliferation was inhibited, the level of hydroxyproline was decreased compared with the control group ($P < 0.01$), and the cell cycle was redistributed when exposed to FFBJRGP *in vitro*.

CONCLUSION: FFBJRGP inhibits hepatic fibrosis *in vivo* and *in vitro*, which is probably associated with downregulation of fibrogenic signal transduction of the TGF-β-Smad pathway.

Key words: Fufang Biejia Ruangan Pill; Hepatic fibrosis; Transforming growth factor-Smad signaling

Core tip: Fufang Biejia Ruangan Pill (FFBJRGP) is the first anti-fibrosis drug approved by the China State Food and Drug Administration. It has been demonstrated that FFBJRGP has a better efficacy of anti-fibrosis. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear. In our study, FFBJRGP showed a strong ameliorative effect in hepatic fibrosis *in vivo* and *in vitro*. It reduced production and deposition of collagen in liver tissues. FFBJRGP inhibited expression of transforming growth factor (TGF-β1) and Smad3, which implied that inhibition of TGF-β/Smad-mediated fibrogenesis may be a central mechanism by which FFBJRGP protects against liver injury.

Yang FR, Fang BW, Lou JS. Effects of Fufang Biejia Ruangan Pills on hepatic fibrosis *in vivo* and *in vitro*. *World J Gastroenterol* 2013; 19(32): 5326-5333 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5326.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5326

INTRODUCTION

Liver fibrosis represents the final common pathway of virtually all chronic liver diseases. It is characterized by the excessive accumulation of extracellular matrix (ECM) and activated hepatic stellate cells (HSCs) that are undergoing myofibroblast transition. Several studies have shown that hepatic fibrosis is a reversible disease, therefore, an effective treatment would probably prevent or reverse the fibrotic process in the liver[1]. In the long pathological progression of hepatic fibrosis to cirrhosis, transforming growth factor (TGF)-β1 is one of the strongest profibrotic cytokines[2,3], and TGF-β-Smad signaling is the main signal transduction pathway[4], which has been verified by several related studies. The downregulation of TGF-β expression and modulation of TGF-β-Smad signaling may be effective in preventing liver fibrosis[5].

Traditional Chinese medicine plays a unique role in the treatment of liver fibrosis. Fufang Biejia Ruangan Pill (FFBJRGP) has been demonstrated to have a better antifibrotic efficacy for its traditional Chinese medical effects of "softening and resolving hard masses, dissolving blood stasis and detoxication, replenishing Qi and Blood". Numerous clinical observations have confirmed that patients with hepatic fibrosis receiving FFBJRGP have a favorable outcome[6]. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear. Thus, in the present study, we investigated the antifibrotic effect and potential mechanisms of action of FFBJRGP in hepatic fibrosis, in order to establish the clinical efficacy and make better application of FFBJRGP.

MATERIALS AND METHODS

Composition of FFBJRGP

The composition of FFBJRGP includes *Carapax Trionycis, Radix Paeoniae Rubra, Radix Angelicae Sinensis, Codonopsis Pilosula* and *Radix Astragali*.

In vivo study

Animals and experiment protocol: Healthy adult Wistar rats, female and male, weighing 237.8 ± 8.5 g, were obtained from the Experimental Animal Center of Academy of Medical Sciences of Chinese People's Liberation Army (Beijing, China). All animals were cared for according to the Guide for the Care and Use of Laboratory Animals (NIH Publications, No. 80-23, revised in 1996). Housed in a room with a 12-h light-dark cycle (temperature 22-24 ℃ and 50%-60% humidity), the rats were given *ad libitum* access to standard laboratory rodent chow and water. All processes conformed to international guidelines on the ethical use of animals.

The rats were subcutaneously injected with carbon tetrachloride (CCl_4) dissolved in peanut oil (CCl_4: peanut oil = 4:6, v/v), 0.5 mL/100 g body weight for the first time, and then 0.3 mL/100 g body weight twice weekly for 8 wk. In the first 2 wk, rats were raised with feedstuff I (80% corn meal, 20% lard, and 0.5% cholesterol). After 2 wk, they were raised with feedstuff Ⅱ (corn meal and 0.5% cholesterol). At the same time, 1 mL 30% alcohol was given orally to each rat every other day from the beginning.

The rats were randomly divided into the normal control group (n = 6); model group (n = 14); FFBJRGP treatment group (n = 12); and colchicine positive control group (n = 12). In the FFBJRGP treatment group, FFBJRGP was administered orally at 0.55 g/kg daily, which was equal to the dose in humans. The rats in the positive control group were given colchicine orally at a daily dose of 0.1 g/kg, which was also equal to the dose for humans. The rats in the normal control and model groups were administered the same volume of physiological saline as for the FFBJRGP group.

Liver laboratory tests: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Serum levels of hyaluronic acid, type Ⅳ collagen, type Ⅲ procollagen and laminin: Serum levels of hyaluronic acid (HA), type Ⅳ collagen (CⅣ), type Ⅲ procollagen (PCⅢ) and laminin (LN) were determined by radioimmunoassay using commercially available kits (Beifang Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions.

Histological examination

Liver tissues were collected from the left lobe of the liver of each rat, and fixed in 15% buffered paraformaldehyde, and dehydrated in a graded alcohol series. Specimens were embedded in paraffin blocks, cut into 5-μm-thick sections and placed on glass slides. The sections were stained with hematoxylin-eosin and Ponceau S[7]. Fibrosis was graded according to the method of Scheuer[8] as follows: stage 0: no fibrosis; stage 1: increase in collagen without formation of septa (small satellite expansion of the portal fields), expansion of portal tracts without linkage; stage 2: formation of incomplete septa not interconnecting with each other, from the portal tract to the central vein; stage 3: complete but thin septa interconnecting with each other, which divide the parenchyma into separate fragments; and stage 4: complete cirrhosis, similar to stage 3 with thicker septa.

Pathological examination was performed by the same pathologist who was blinded to the treatment assignment for the rats.

Determination of TGF-β1 mRNA level in liver tissues by real-time reverse transcriptase-polymerase chain reaction: Total RNA was extracted from liver tissues of each group with the tissue/cell total RNA isolation kit according to the manufacturer's protocol (Dalian TaKaRa Biotechnology Company, Dalian, China). The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer. Total RNA was reversibly transcribed into cDNA using the cDNA synthesis kit according to the manufacturer's protocol (Dalian TaKaRa Biotechnology Company, Dalian, China). The ABI PRISM 7900 HT Real Time-polymerase chain reaction (PCR) System and real-time PCR kit were used according to the manufacturers' instructions. The specific primers for the target gene and β-actin were synthesized by Dalian TaKaRa Biotechnology Company (Dalian, China), as follows: TGF-β1: 5'-TGGCGTTACCTTGGTAACC-3' (forward); 5'-GGTGTT GAGCCCTTTCCAG-3' (reverse); β-actin: 5'-ACCCTTAAGGCCAACCGTGA AAAG-3' (forward); 5'-TCATGAGGTAGTCTGTCAGGT-3' (reverse).

The two-step PCR procedure was as follows: predenaturation for 30 s at 95 ℃, 1 cycle; 94 ℃ for 15 s and 56 ℃ for 40 s, 40 cycles. The final products were identified by electrophoresis in 1.5% agarose gel and melt curve analysis. Melt curve detection: 95 ℃ for 15 s, 60 ℃ for 15 s, and 95 ℃ for 15 s. The final results were described with the relative values ($2^{-\Delta\Delta Ct}$). The calculation and analysis were performed by Sequence Detection Software version 2.1 in the ABI PRISM 7900 HT Real Time PCR System.

Determination of Smad3 level in liver tissues by Western blotting: Total protein was extracted from liver tissues and analyzed with bicinchoninic acid protein concentration assay kit. Sample protein was separated by electrophoresis in 12% SDS-PAGE with a Bio-Rad electrophoresis system (Hercules, CA, United States). The primary antibodies (rabbit anti Smad3 antibody, 1:1000 dilution) were incubated at 4 ℃ overnight. The corresponding horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:5000 dilution) were incubated at room temperature. Immobilon Western chemiluminescent horseradish peroxidase substrate and Quantity ONE were used for revealing and quantitative analysis of the blots. β-actin was used as the internal control.

In vitro study

Drug serum preparation: The normal rats were administered with FFBJRGP and colchicine at a dose of 0.55 and 0.1 g/kg, respectively, for 2 d. At 2 h after the final administration, the sera were collected from the rats, mixed, and inactivated at 56 ℃ for 30 min. The blank control sera were collected from the normal rats.

Cell culture: HSC-LX-2 cells, an immortalized human HSC line, were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cultures were placed in a humidified atmosphere of 5% CO_2 at 37 ℃, and the medium was changed twice a week.

Cell viability test: HSC-LX-2 cells were seeded into 96-well plates at a density of 2×10^4 cells/well until 50% confluence. Cells treated with the above drug sera (20 μL/well) for 48 h were incubated with 5 mg/mL methyl thiazolyl tetrazolium (MTT) in DMEM for 4 h at 37 ℃. The supernatant was removed and 100 μL DMSO was added to each well to dissolve the formazan product. Absorbance at 570 nm was measured using a microplate reader.

Determination of hydroxyproline content: Collagen was determined by estimating the hydroxyproline content, an amino acid characteristic of collagen. HSC-LX-2 cells were lysed after treatment with the above drug sera. The lysates were used to measure hydroxyproline content using commercially available kits according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China).

Cell cycle analysis: For cell cycle analysis, HSC-LX-2 cells were synchronized by serum starvation in medium containing 0.4% serum for 24 h and induced to re-enter the cell cycle by an exchange of DMEM supplemented with 10% FBS. Drug sera of different groups were added (1 mL/bottle); the cells were cultured for 48 h and then harvested; washed and suspended in phosphate-buffered saline (PBS) twice; fixed in 80% ethanol for 48 h at 4 ℃; and suspended in 500 μL PBS containing RNase A for 30 min at 37 ℃. A total of 2×10^6 cells were harvested and resuspended in 0.5 mL of a solution containing 50 μg/mL propidium iodide, 1 mg/mL sodium citrate, 100 μg/mL RNase, and 0.1% Triton X-100. Flow cytometric analysis was made with a fluorescence-activated cell sorter. Forward light scatter characteristics were used to exclude cell debris from the analysis. The G0/G1 and S phases of the cell cycle were analyzed by diploid staining profiles.

Statistical analysis

All values were expressed as mean ± SD. Comparisons were analyzed by one-way ANOVA using the SPSS 12.0 statistical package. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of FFBJRGP on liver function

There were significant differences in the ALT and AST

Table 1 Effect of Fufang Biejia Ruangan Pill on serum levels (mean ± SD)

Group	ALT(U/L)	AST(U/L)	PCⅢ (μg/mL)	HA (ng/mL)	LN (ng/mL)	CIV (ng/mL)
Control	23.8 ± 8.5[b]	30.0 ± 11.4[b]	15.16 ± 15.12[b]	205.30 ± 48.92[a]	82.02 ± 8.86	21.71 ± 1.76
Model	62.0 ± 23.7	98.8 ± 40.0	35.73 ± 17.90	563.82 ± 335.54	89.57 ± 7.59	29.20 ± 6.17
FFBJRGP-treated	50.02 ± 3.7	57.2 ± 30.0[b]	30.18 ± 9.41	456.18 ± 410.83	85.46 ± 7.51	28.02 ± 9.45
Colchicine-treated	46.1 ± 14.8	66.0 ± 33.2[a]	34.08 ± 9.19	313.17 ± 230.06[a]	88.61 ± 8.97	29.22 ± 7.95

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PCⅢ: Type Ⅲ procollagen; HA: Hyaluronic acid; LN: Laminin; CIV: Type Ⅳ collagen; FFBJRGP: Fufang Biejia Ruangan Pill.

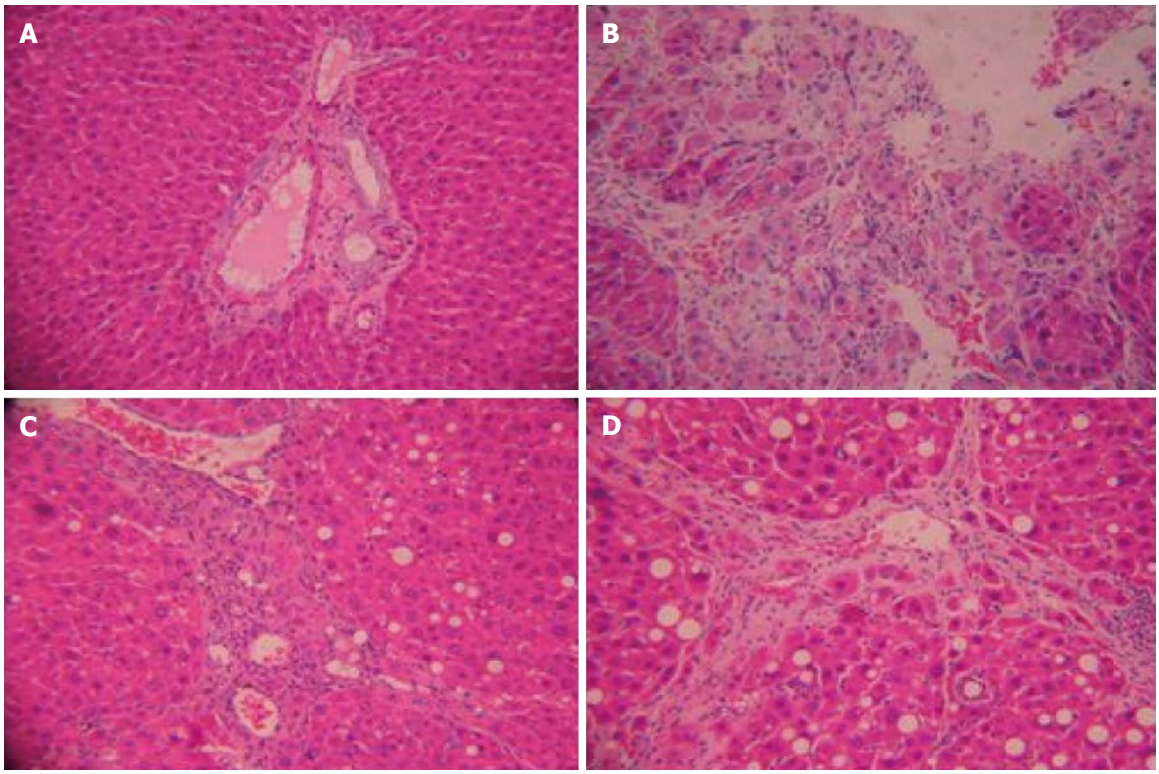

Figure 1 Histological profiles of liver tissues in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats (stained with hematoxylin and eosin, × 100).

activities among the experimental groups. The ALT and AST activities in the model group were significantly higher compared with those in the normal control group ($P < 0.01$), while those in the FFBJRGP-treated group (0.55 g/kg) were significantly lower than in the model group ($P < 0.01$), and those in the colchicine-treated group (0.1 g/kg) were also lower than in the model group ($P < 0.05$) (Table 1).

Effect of FFBJRGP on serum levels of PCⅢ, HA, LN and CIV

The serum levels of PCⅢ, HA, LN and CIV were significantly increased in the model group, as serum markers of hepatic fibrosis, when compared with the normal control group. The FFBJRGP-treated (0.55 g/kg) and colchicine-treated (0.1 g/kg) groups had decreased serum levels of PCⅢ, HA, LN and CIV (Table 1).

Effect of FFBJRGP on hepatic histopathology

At the end of the study, normal hepatic lobules, without fibroplasia and inflammatory cell infiltration, were observed in normal rats (Figure 1A). Many inflammatory cells infiltrated the intra- and inter-lobular areas, and cell degeneration, focal necrosis and bile duct proliferation were found in rats with hepatic fibrosis (Figure 1B). The histological pattern of the livers treated by FFBJRGP showed a low level of infiltration of leukocytes, necrosis, and bile duct proliferation (Figure 1C). Similar trends were also observed in the colchicine group (Figure 1D).

Effect of FFBJRGP on hepatic collagen deposition

The rat liver was stained with Ponceau S, which showed the collagen fibers as red. Normal hepatic lobules without fibroplasia were observed in normal rats. Complete septa interconnecting with each other were formed, which divided the parenchyma into separate fragments in the model group. The rats treated with FFBJRGP and colchicine had less pronounced destruction of the liver architecture, with decreased collagen deposition (Table 2, Figure 2).

Table 2 Liver histopathological semiquantitative scores (mean ± SD)

Group	*n*	Scores					Staging scores
		0	I	II	III	IV	
Control	6	6					0.00 ± 0.00[b]
Model	13				2	11	26.08 ± 5.85
FFBJRGP-treated	9				4	5	20.33 ± 6.12[b]
Colchicine-treated	9		1	3	5	2	19.00 ± 6.38[b]

[b]$P < 0.01$ *vs* model group. FFBJRGP: Fufang Biejia Ruangan Pill.

Effect of FFBJRGP on TGF-β1 and Smad3 expression in liver

The expression of TGF-β1 and Smad3 in the rat liver was quantified. Expression of TGF-β1 was twofold higher in the model group than in the normal control group. FFBJRGP and colchicine therapy significantly decreased TGF-β1 expression (Table 3). Compared with the normal control group, the expression of Smad3 in the model group was increased ($P < 0.01$). Compared with the model group, expression of Smad3 was decreased in the FFBJRGP and colchicine groups (Table 3, Figure 3).

FFBJRGP significantly suppresses HSC-LX-2 cell proliferation

The antiproliferative activity in HSC-LX-2 cells was determined by cell viability using the MTT assay. HSC-LX-2 cell proliferation was inhibited by FFBJRGP. Compared with the blank group (100%), FFBJRGP at a dose of 0.55 g/kg inhibited HSC-LX-2 cell proliferation by 31%, and colchicine at a dose of 0.1 g/kg inhibited proliferation by 28%. The antiproliferative effects were not related to the nonspecific cytotoxic effects of FFBJRGP because cells showed normal morphology.

FFBJRGP significantly reduces hydroxyproline content

To assess the effect of FFBJRGP on ECM production in HSC-LX-2 cells, hydroxyproline content was examined. Hydroxyproline content was decreased in the FFBJRGP group (1.78 ± 0.06 μg/mL, $P < 0.01$) compared with the blank group (2.35 ± 0.12 μg/mL), and it was also decreased in the colchicine group (1.91 ±0.14 μg/mL, $P < 0.01$).

Effect of FFBJRGP on cell cycle

Flow-cytometric assays were carried out to evaluate the effect of FFBJRGP on the cell cycle of activated HSC-LX-2 cells. Compared with the blank control, FFBJRGP altered the percentage of cells in the G_0/G_1 and S phases. The percentage of cells in the G_0/G_1 phase was increased in the FFBJRGP group (52.6% ± 1.2%, $P < 0.01$) compared with the blank group (46.7% ± 0.0%), and the percentage of cells in S phase was decreased in the FFBJRGP group (34.9% ± 7.9%) compared with the blank group (42.1% ± 0.5%). However, the change in the percentage of cells in the G_0/G_1 and S phases was not obvious in the colchicine group.

DISCUSSION

Hepatic fibrosis is thought to be a reversible disease, however, at present there is no satisfactory method in clinical practice to reverse the pathological process. Several drugs, including antisense TGF-β receptor, cytokines[9], antioxidants, chemical drugs, soluble type Ⅱ receptor of TGF-β1, and TGF-β1 antibody have been used to block experimental hepatic fibrosis, but their effects are not as promising as we expected. Besides, some traditional Chinese drugs are effective in preventing fibrogenesis and other causes of chronic liver injury[10], and this offers more hope for the future control of liver fibrosis and cirrhosis[11]. These drugs have the advantages of being cheap, safe and easy to acquire, but most of them are limited in animal experiments and clinical observation, and systematic study at molecular level is lacking.

The activation of HSCs by cytokines is considered to be of importance during the long duration of liver fibrosis. These activated HSCs then become the main source of most cytokines and collagen. Among the cytokine-mediating factors, TGF-β1 is an essential profibrogenic factor[12-17]. In addition, the TGF-β-Smad signaling pathway is the main pathway of TGF-β1[18-20], which transfers the stimulating signal from outside into the affected cells. The Smad proteins consist of a large family of transcription factors, which are also found in vertebrates, insects and nematodes. To date, Smads are the only TGF-β receptor substrates with the ability to propagate signals. Two different transmembrane protein serine/threonine kinases, named as TGF-β receptor type Ⅰ and Ⅱ, are brought together by the ligand, which acts as a receptor assembly factor[21]. Before this occurs, receptor Ⅰ is inactive because a wedge-shaped GS region is inserted into the kinase domain, dislocating the catalytic center. During TGF-β signal transduction, receptor Ⅱ is activated firstly. TGF-β and its receptor then form an activated complex. In the ligand-induced complex, activated receptor Ⅱ phosphorylates the GS region of receptor type Ⅰ, resulting in the activation of the receptor Ⅰ kinase. The type Ⅰ receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads), which are Smad 2 and Smad 3[22]. R-Smads are activated and form a complex consisting of R-Smads and Smad 4, which belongs to Co-Smad. The Smads complex accumulates in the nucleus. This procedure leads to the formation of the functional transcriptional complexes. The R-Smads and Co-Smads in this complex may participate in DNA binding and recruitment of transcriptional cofactors[23]. CREB binding protein is the main downstream molecule and the general transcriptional coactivator. After transfer into the nucleus, the transcriptional complex binds to the certain domain of the target gene and causes gene expression, such as collagen production. Excess collagen production

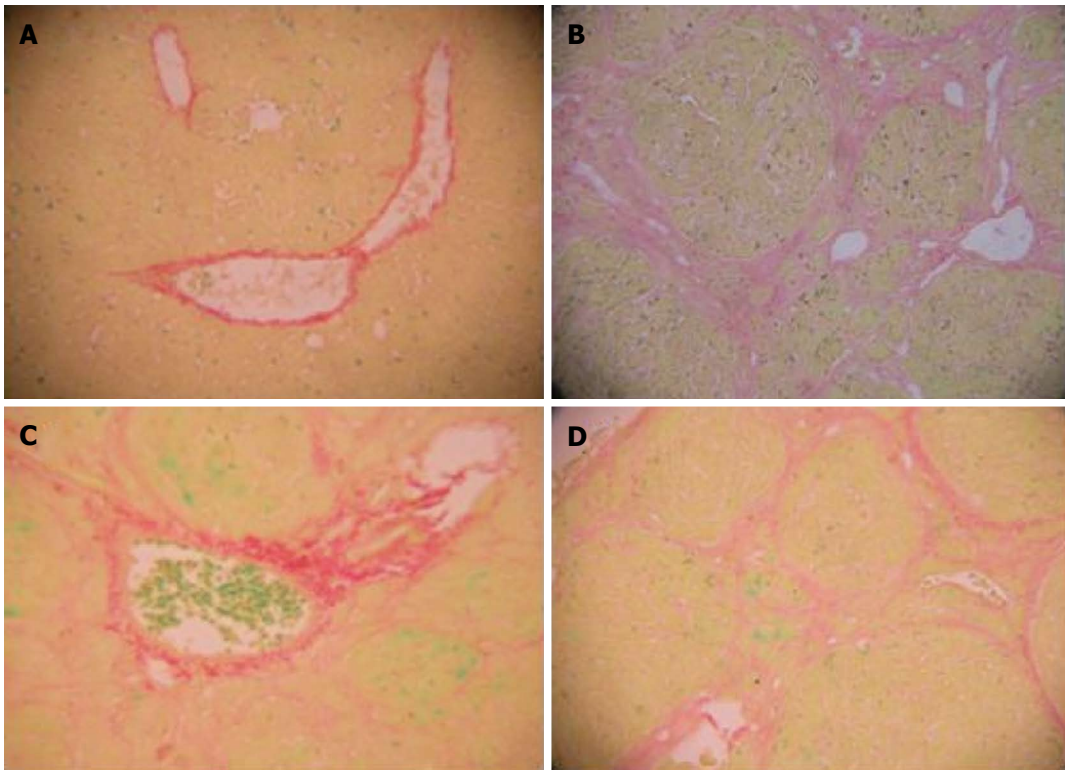

Figure 2 Profiles of liver tissues in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats (stained with Ponceau S, × 100).

Table 3 Expression of transforming growth factor-β1 and Smad3 (mean ± SD)

Groups	TGF-β1/β-actin	Smad3/β-actin
Control	1.00 ± 0.00[b]	0.62 ± 0.08[b]
Model	3.29 ± 2.08	1.33 ± 0.10
FFBJRGD-treated	2.08 ± 0.57	0.95 ± 0.12[b]
Colchicine-treated	2.25 ± 0.82	1.15 ± 0.06[b]

[b]$P < 0.01$ *vs* model group. TGF-β: Transforming growth factor-β; FFBJRGP: Fufang Biejia Ruangan Pill.

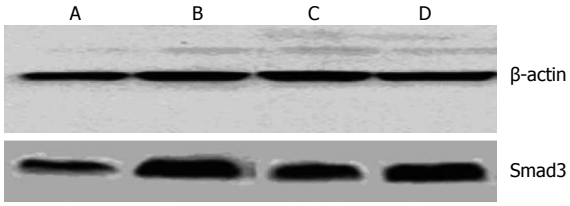

Figure 3 Western blotting for Smad3 expression in rats. A: Normal rats; B: Rats with hepatic fibrosis; C: Fufang Biejia Ruangan Pill-treated rats; D: Colchicine-treated rats.

leads to collagen deposition in liver tissues and eventually hepatic fibrosis or cirrhosis. The TGF-β-Smad signaling pathway is important in the formation of hepatic fibrosis, therefore, blocking its transduction may inhibit hepatic fibrosis. Inhibition of the TGF-β-Smad signaling pathway or modulating the gene expression of certain Smads can interfere with hepatic fibrosis[24].

Hepatic fibrosis is characterized by abnormal accumulation of ECM proteins, particularly collagen. The main collagen-producing cells in the liver are HSCs, which proliferate and undergo a process of activation during the development of fibrosis, resulting in increased capacity for collagen synthesis. A simple and reproducible tool is necessary to assess accurately the degree of hepatic fibrosis in clinical practice.

According to the theory of traditional Chinese medicine, hepatic fibrosis is characterized by internal damp (Shi), heat (Re), poison (Du), blood stasis (Yu), and both Qi and Yin asthenia[25,26]. In the present study, not only CCl_4, but also cholesterol, lard and alcohol were used to establish a model of hepatic fibrosis. CCl_4 is poison, and cholesterol, lard and alcohol produce damp and heat, which cause healthy energy asthenia, blood stasis exacerbation, unrelievable damp and heat, and induce hepatic fibrosis. This model well simulates these symptoms. The serum markers of ECM have been used for the assessment of hepatic fibrosis because they are neither invasive nor unavailable. Serum levels of CⅣ, PCⅢ, HA and LN are positively correlated with the inflammatory activity and degree of hepatic fibrosis. Hydroxyproline content in the liver is considered another index of collagen metabolism and provides valuable information about the biochemical and pathological states of liver fibrosis. The present study demonstrated that consumption of FFBJRGP prevented the development of hepatic fibrosis in a rat model of CCl_4-induced liver fibrosis. The results were confirmed by both liver histology and quantitative measurement of serum levels of CⅣ, PCⅢ, HA and LN.

Accordingly, inhibition of proliferation, and reduced collagen content, were also observed in activated HSC-LX-2 cells following FFBJRGP treatment. We also found that FFBJRGP downregulated the expression of TGF-β1 and Smad3, and altered the percentage of cells in the G_0/G_1 and S phases.

In conclusion, the traditional Chinese medicine FFBJRGP shows significant antifibrotic effects. Inhibiting activation of TGF-β/Smad signaling may be an underlying mechanism by which FFBJRGP protects against chronic liver disease associated with fibrosis.

COMMENTS

Background

In China, the incidence of hepatic cirrhosis is still high. Hepatic cirrhosis develops from fibrosis. If treated properly at the fibrosis stage, cirrhosis can be prevented. Fufang Biejia Ruangan Pill (FFBJRGP), a Chinese medical product, is used extensively for the treatment of hepatic fibrosis. FFBJRGP has better antifibrotic efficacy due to its effects of "softening and resolving hard masses, dissolving blood stasis and detoxication, replenishing Qi and Blood" in the philosophy of traditional Chinese medicine. However, the underlying therapeutic mechanisms of FFBJRGP in hepatic fibrosis are still unclear, even though it has become the best-selling traditional Chinese medicine. Thus, in the present study, the authors investigated the antifibrotic effect and potential mechanisms of action of FFBJRGP in hepatic fibrosis, in order to establish the clinical efficacy and make better application of FFBJRGP.

Research frontiers

Recent research shows that hepatic fibrosis can be reversed by regulating collagen metabolism, inhibiting the activation of hepatic stellate cells (HSC), or by promoting HSC apoptosis. Hepatic extracellular matrix mainly results from HSCs, which can be activated by the fibrogenesis signaling pathway.

Innovations and breakthroughs

This study confirmed that FFBJRGP can inhibit hepatic fibrosis *in vivo* and *in vitro*. FFBJRGP can improve liver function, inhibit collagen deposition, alleviate hepatic injury, inhibit HSC-LX-2 cell proliferation, and redistribute the cell cycle, which is probably associated with its downregulation of the fibrogenic transforming growth factor (TGF)-β-Smad signaling pathway.

Applications

FFBJRGP can inhibit hepatic fibrosis *in vivo* and *in vitro*, which implies it is a good drug for patients with hepatic fibrosis. This study provides scientific data for its better application.

Terminology

FFBJRGP is a Chinese medicine that can inhibit hepatic fibrosis. HSCs are key cells that can produce a considerable amount of extracellular matrix and promote collagen deposition. TGF-β1-Smads is a fibrogenic signal transduction pathway that can activate HSCs and promote collagen synthesis.

Peer review

This study describes the antifibrogenic effects of the Chinese herbal medicine FFBJRGP. The data strongly suggests that FFBJRGP may be therapeutically useful in patients with hepatic fibrosis.

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P- Reviewers Butterworth J, Elena V, Jun DW
S- Editor Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5334

World J Gastroenterol 2013 August 28; 19(32): 5334-5339
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Prevalence and features of fatty liver detected by physical examination in Guangzhou

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Supported by The Science and Technology Project of Guangdong Province, China, No. 2010B031600047
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Received: February 26, 2013 Revised: July 7, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

AIM: To investigate the prevalence of fatty liver discovered upon physical examination of Chinese patients and determine the associated clinical characteristics.

METHODS: A total of 3433 consecutive patients who received physical examinations at the Huangpu Division of the First Affiliated Hospital at Sun Yat-sen University in Guangzhou, China from June 2010 to December 2010 were retrospectively enrolled in the study. Results of biochemical tests, abdominal ultrasound, electrocardiography, and chest X-ray were collected. The diagnosis of fatty liver was made if a patient met any two of the three following ultrasonic criteria: (1) liver and kidney echo discrepancy and presence of an increased liver echogenicity (bright); (2) unclear intrahepatic duct structure; and (3) liver far field echo decay.

RESULTS: The study population consisted of 2201 males and 1232 females, with a mean age of 37.4 ± 12.8 years. When all 3433 patients were considered, the overall prevalence of hyperlipidemia was 38.1%, of fatty liver was 26.0%, of increased alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) levels was 11.9%, of gallstone was 11.4%, of hyperglycemia was 7.3%, of hypertension was 7.1%, and of hyperuricemia was 6.2%. Of the 2605 patients who completed the abdominal ultrasonography exam, 677 (26.0%) were diagnosed with fatty liver and the prevalence was higher in males (32.5% *vs* females: 15.3%, $P < 0.001$). The overall prevalence of fatty liver increased with age, with the peak prevalence (39.5%) found in the 60 to 70-year-old age group. Among patients between the ages of 18 to 50-year-old, the prevalence of fatty liver was significantly higher in males (20.2% *vs* females: 8.7%, $P < 0.001$); the difference in prevalence between the two sexes in patients > 50-year-old did not reach statistical significance. Only 430 of the patients diagnosed with fatty liver had complete information; among those, increased ALT and/or AST levels were detected in only 30%, with all disturbances being mild or moderate. In these 430 patients, the overall prevalence of hypertriglyceridemia was 31.4%, of mixed type hyperlipidemia was 20.9%, of hypercholesterolemia was 12.3%, of hyperglycemia was 17.6%, of hypertension was 16.0%, of hyperuricemia was 15.3%, and of gallstone was 14.4%. Again, the prevalences of hypertriglyceridemia and hyperuricemia were higher in males (hypertriglyceridemia, 36.0% *vs* females: 12.0%, $P < 0.05$; hyperuricemia, 17.3% *vs* females: 7.2%, $P < 0.05$); in contrast, however, the prevalences of mixed type hyperlipidemia and hypercholesterolemia was higher in females (mixed type hyperlipidemia, 18.7%

vs females: 30.1%, $P < 0.05$, hypercholesterolemia, 9.5% *vs* females: 24.1%, $P < 0.05$). Finally, comparison of the fatty liver group to the non-fatty liver group showed that prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia were higher in the former (all $P < 0.01$).

CONCLUSION: A high prevalence of fatty liver is detected upon physical examination in Guangzhou, and the primary associated clinical findings are hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia.

Key words: Fatty liver; Nonalcoholic; Prevalence; Hyperlipidemia; Hyperglycemia; Hypertension

Core tip: This study represents the first published investigation of fatty liver prevalence detected by routine physical examinations of individuals residing in the Huangpu District of Guangzhou, China. A high prevalence of fatty liver (26.0%) was detected among the total physical examinees and was characterized by an age-related increasing trend, with the highest prevalence (39.5%) found among individuals between 60 and 70-year-old. The individuals diagnosed with fatty liver also showed significantly higher prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia than their non-fatty liver counterparts (all $P < 0.01$), suggesting a close association between fatty liver and dysmetabolic factors.

Liao XH, Cao X, Liu J, Xie XH, Sun YH, Zhong BH. Prevalence and features of fatty liver detected by physical examination in Guangzhou. *World J Gastroenterol* 2013; 19(32): 5334-5339 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5334.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5334

INTRODUCTION

Prevalence of fatty liver in China has risen consistently over recent years, accompanying improvements in people's living conditions and adoption of a more Westernized diet. In Western countries, estimates of fatty liver prevalence in the adult population have ranged from 20% to 33%[1], and the most recent prevalence estimate reported for Shanghai, China is 20.82%[2]. In addition to being the most frequently diagnosed liver disease in Chinese clinics, fatty liver represents a particularly alarming threat to human health and public healthcare systems as it can readily progress to steatohepatitis, cirrhosis, or liver cancer.

To gain further understanding about the prevalence and presenting features of fatty liver in China, the current study was designed as a single-site retrospective analysis of adult patients who underwent physical examinations in Guangzhou and were diagnosed with fatty liver.

MATERIALS AND METHODS

Study participants

A total of 3433 consecutive adult patients who underwent routine physical examinations at the Huangpu Division of the First Affiliated Hospital of Sun Yat-sen University from June 2010 to December 2010 were retrospectively enrolled in the study.

Physical examination

Patients presented to the hospital for blood sampling after 10 h of fasting; all serological measurements were carried out on-site at the certified laboratory. Automated techniques (Architect C8000 automatic biochemistry analyzer; Abbott Laboratories, Abbott Park, IL, United States) were used to measure plasma concentrations of glucose, total cholesterol (CHOL), triglyceride (TG), serum uric acid, serum creatinine, blood urea nitrogen, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Direct sandwich enzyme-linked immunosorbent assay was used to measure hepatitis B virus markers.

Abdominal ultrasonography was performed to detect the presence of fatty infiltration in the liver, using standard imaging criteria to assess hepatic fat[3]. Electrocardiography and chest X-ray were performed to rule out serious heart and lung diseases.

Diagnostic criteria and definitions

Hypertension was diagnosed by a systolic pressure of $\geqslant$ 140 mmHg and/or a diastolic pressure of $\geqslant$ 90 mmHg, according to the 2010 Chinese guidelines for the management of hypertension[4]. Hyperglycemia was diagnosed by fasting plasma glucose level of $\geqslant$ 110 mg/dL[5]. Hyperuricemia was diagnosed by blood uric acid level of $\geqslant$ 7 mg/dL in men and $\geqslant$ 6 mg/dL in women[6]. Abnormal serum creatinine level was defined as $\geqslant$ 1.6 mg/dL. The various types of hyperlipidemia were diagnosed by CHOL $\geqslant$ 200 mg/dL and TG $\geqslant$ 150 mg/dL for mixed type hyperlipidemia, CHOL $\geqslant$ 200 mg/dL and TG < 150 mg/dL for hypercholesterolemia, and CHOL < 200 mg/dL and TG $\geqslant$ 150 mg/dL for hypertriglyceridemia[7]. Fatty liver was diagnosed when a patient met any two of the three following ultrasonic criteria: liver and kidney echo discrepancy and presence of increased liver echogenicity (bright); unclear intrahepatic duct structure; liver far field echo decay[3].

Statistical analysis

All statistical analyses were performed by the SPSS statistical software suite, version 13.0 (Chicago, IL, United States). All reported *P*-values were two-sided, and $P < 0.05$ was considered statistically significant. Descriptive data are expressed as mean ± SD. Comparisons between quantitative data were carried out by the Student's *t*-test, and comparisons between categorical variables were carried out by the χ^2 test.

Table 1 Prevalence of dysmetabolic diseases and biochemical abnormalities in the total study population *n* (%)

	Patients examined	Positive patients
Hyperlipidemia	2715	1035 (38.1)
Fatty liver	2605	677 (26.0)
Increased ALT and/or AST levels	3393	405 (11.9)
Gallstone	2605	296 (11.4)
HBsAg	2244	198 (8.8)
Hyperglycemia	2767	203 (7.3)
Hypertension	2938	210 (7.3)
Hyperuricemia	2700	167 (6.2)
Increased Scr levels	2150	10 (6.2)

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

RESULTS

Demographic and clinical characteristics of physical examinees

The study population of physical examinees consisted of 2201 males and 1232 females. Three-thousand-two-hundred-and-five of the patients described themselves as employed, with the majority being mental laborers and a small percentage being physical laborers (84.8% and 15.2%, respectively). The mean age of the overall study population was 37.4 ± 12.8-year-old (range: 18-87 years). The overall prevalences of dysmetabolic diseases and perturbed biochemical findings are listed in Table 1.

Characteristics of the 677 patients diagnosed with fatty liver

Of the 2605 subjects who underwent abdominal ultrasonography, 677 subjects (26.0%) showed imaging signs of fatty liver. The prevalence of fatty liver was significantly higher in males than in females (32.5% *vs* 15.3%, $P < 0.001$). The overall prevalence of fatty liver increased with age, with the 60 to 70-year-old age group representing the peak prevalence (39.5%) and without age bias. However, when the larger age group of 18 to 50-year-old was considered, a significantly higher prevalence was found for males (20.2% *vs* females: 8.7%, $P < 0.001$); this trend did not exist for the > 50-year-old age group (34.6% *vs* females: 38.9%, $P = 0.301$) (Table 2). The overall prevalences of dysmetabolic diseases and perturbed biochemical findings for the 677 patients with fatty liver are listed in Table 3.

Characteristics of the 430 patients with fatty liver and complete physical examination data

In total, 430 of the patients diagnosed with fatty liver also had complete data accounting for all of the physical examination components; this group was comprised of 347 males (80.7%) and 83 females (19.3%), with a mean age of 43.8 ± 12.4-year-old (range: 19-78 years). One-hundred-and-twenty-nine patients (30.0%) showed mildly or moderately increased ALT and/or AST levels (40-200 U/L); however, the amount of patients with increased ALT was significantly higher than of patients with increased AST (29.8% *vs* 7.9%, $\chi^2 = 67.2$, $P < 0.001$). The majority of patients with abnormal ALT and/or AST levels showed a mild increase, and included 102 patients (70.1%) with ALT and/or AST levels < 2-times the upper normal limit and 27 patients (20.9%) with ALT and/or AST levels 2 to 5-times the upper normal limit. Males were more likely to have increased ALT and/or AST levels (32.6% *vs* 19.3%, $P < 0.05$).

The overall prevalences of dysmetabolic diseases and perturbed biochemical findings for the 430 patients with fatty liver and complete data are listed in Table 4. The prevalences of hypertriglyceridemia and hyperuricemia were higher in males than in females, but the prevalences of mixed type hyperlipidemia and hypercholesterolemia were higher in females than in males ($P < 0.05$). The difference in the prevalence of hypertension or hyperglycemia between males and females did not reach statistical significance ($P > 0.05$).

Risk factors associated with fatty liver

Patients with no signs of fatty liver and hepatitis B surface antigen negativity were assigned to a non-fatty liver group, which included 382 males (77.2%) and 113 females (22.8%), with a mean age of 42.5 ± 10.9-year-old. Compared to the fatty liver group, the sex and age distributions were not significantly different ($P > 0.05$). As shown in Table 5, the prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia were significantly higher in the fatty liver group than in the non-fatty liver group ($P < 0.01$).

DISCUSSION

Fatty liver is a clinicopathologic syndrome that manifests from hepatic steatosis and excessive fat accumulation caused by a variety of factors. The syndrome spectrum includes simple fatty liver, steatohepatitis, fatty liver cirrhosis, and associated hepatocellular carcinoma. While liver biopsy is the gold standard for diagnosis of fatty liver, ultrasonography is generally used as a non-invasive screening method for the general population. The reported estimates of fatty liver cases diagnosed by ultrasonography have ranged from 17% to 46% in Europe, United States and other Asian countries[8-11]. In the current survey of physical examinees in Huangpu District, Guangzhou, the prevalence of fatty liver detected in physical examination was 26%; this rate is similar to previous estimates made in other Chinese cities[12,13].

It is generally recognized that the prevalence of fatty liver increases with age, with the highest rates found in the age group of 50 to 70-year-old[10,14]. In the present study, the highest prevalence of fatty liver occurred in the age group of 60 to 70-year-old. It is important to note that elderly people harbor significantly more of the known risk factors for fatty liver, such as obesity, hypertension, diabetes, and hyperlipidaemia. Furthermore, aging brings restrictions on physical mobility, which in turn supports or promotes the above risk factors and can eventually lead to a higher prevalence of fatty liver[15].

Table 2 Age and sex distribution of overall fatty liver prevalence

Age (yr)	All patients	Patients with fatty liver	Overall prevalence	Prevalence in males	Prevalence in females	χ^2	*P* value
18-19	17	1	5.80%	7.10%	0.00%	-	-
20-29	1055	55	5.20%	6.40%	2.30%	7.47	0.006
30-39	1003	206	20.50%	26.70%	8.70%	44.80	< 0.001
40-49	799	212	26.50%	34.50%	16.80%	31.78	< 0.001
50-59	331	123	37.20%	35.90%	39.00%	0.32	0.569
60-69	147	58	39.50%	37.80%	42.10%	0.27	0.601
⩾ 70	81	22	27.10%	24.50%	32.10%	0.54	0.464
Total	2605	677	26.00%	32.50%	15.30%	95.18	< 0.001

Table 3 Prevalence of dysmetabolic diseases and biochemical abnormalities in the 677 patients diagnosed with fatty liver *n* (%)

	Patients examined	Positive patients
Increased ALT and/or AST levels	676	199 (29.4)
Hypertriglyceridemia	635	197 (31.0)
Mixed type hyperlipidemia	635	135 (21.3)
Hypercholesterolemia	635	92 (14.5)
Hyperglycemia	640	116 (18.1)
Hypertension	636	102 (16.0)
Gallstone	677	97 (14.4)
Hyperuricemia	627	87 (13.9)
HBsAg	506	39 (7.7)
Increased Scr levels	507	3 (0.6)

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

Table 4 Sex distribution of prevalence of dysmetabolic diseases and biochemical abnormalities in the 430 subjects with fatty liver *n* (%)

	All patients	Male patients	Female patients	χ^2	*P* value
Increased ALT and/or AST levels	129 (30.0)	113 (32.6)	16 (19.3)	5.63	0.018
Hypertriglyceridemia	135 (31.4)	125 (36.0)	10 (12.0)	17.87	< 0.001
Mixed type hyperlipidemia	90 (20.9)	65 (18.7)	25 (30.1)	5.25	0.022
Hypercholesterolemia	53 (12.3)	33 (9.5)	20 (24.1)	13.19	< 0.001
Hyperglycemia	76 (17.6)	59 (17.0)	17 (20.5)	0.56	0.455
Hypertension	69 (16.0)	53 (15.3)	16 (19.3)	0.80	0.372
Hyperuricemia	66 (15.3)	60 (17.3)	6 (7.2)	5.22	0.022
Gallstone	62 (14.4)	50 (14.4)	12 (14.4)	0.00	0.991
HBsAg	34 (7.9)	27 (7.9)	7 (7.9)	0.04	0.843
Increased Scr levels	3 (0.7)	3 (0.9)	0 (0.0)	-	-

HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Scr: Serum creatinine.

Most studies have shown that men are more likely to develop fatty liver than women before the age of 50 years, but both sexes face a similar likelihood of developing the condition after 50[16,17]. Similarly, a study involving 26527 Chinese subjects who underwent routine health check-ups showed that the prevalence of fatty liver was 31% in men and 16% in women[18].

The significant difference in the prevalence of fatty liver between men and women before the age of 50 is probably a result of the clear differences in the amount and distribution of body fat between the sexes. Men usually store fat in the abdomen whereas women tend to store fat in the subcutaneous tissue. While the reasons for this differential fat accumulation in men and women remain unclear, evidence from cell research have suggested that lipid metabolism pathways may play important roles. Moreover, molecular studies have uncovered distinctions between men and women in the activity and metabolism of lipids. A Japanese study showed that the triglyceride and cholesterol particles were larger in men than those in women, and both of these factors are associated with risk for fatty liver[15,19].

The fact that the significant difference in prevalence of fatty liver among men and women is lost after the age of 50 is intriguing. Women of this age have decreased adiponectin levels, as a result of the lower estrogen and higher androgen that occur after menopause[20,21]; the resetting of postmenopausal women's physiology to that which more closely resembles the male physiology may account for the similar prevalence of fatty liver between the sexes at this age. The Chinese study mentioned above also showed that the mean ALT levels in men were significantly higher than those in women before 50; yet, the peak levels of ALT were observed in women older than 50 years, which might be related to menopause changes and the decreased physical exercise that frequently accompanies this period of life[18]. Nonetheless, these previously published findings, along with ours presented herein, highlight the importance of prevention and screening of fatty liver in men and postmenopausal women.

Most patients with fatty liver are diagnosed without or with mild clinical symptoms. In a study of fatty liver clinical characteristics by Powell *et al*[22], 79% of diagnosed patients were shown to have normal serum transaminase levels. In the current study, 30% of the patients diagnosed with fatty liver presented with mildly increased ALT and/or AST levels, and most of those were accounted for by ALT increase. Thus, the most common type of fatty liver was nonalcoholic fatty liver disease (NAFLD). Moreover, the fatty liver group showed higher prevalences of hyperlipidemia, hyperglycemia, hypertension, and hyperuricemia as compared to patients without fatty liver, suggesting that fatty liver may be closely associated with these disorders.

Risk factors known to be associated with NAFLD include metabolic syndrome, diabetes, and obesity. Prevalence estimates of NAFLD have ranged from 40% to 70% in patients with type 2 diabetes mellitus (T2DM)[23],

Table 5 Distribution of prevalence of risk factors in patients with and without fatty liver

	Patients with fatty liver (n = 430)		Patients without fatty liver (n = 495)		χ^2	P value
	All patients	Prevalence	All patients	Prevalence		
Hypertriglyceridemia	135	31.40%	45	9.10%	73.04	< 0.001
Mixed type hyperlipidemia	90	20.90%	33	6.70%	40.61	< 0.001
Hypercholesterolemia	53	12.30%	95	19.20%	8.07	0.004
Hyperglycemia	76	17.70%	25	5.00%	37.70	< 0.001
Hypertension	69	16.00%	32	6.50%	21.72	< 0.001
Hyperuricemia	66	15.30%	14	2.80%	45.66	< 0.001

57%-74% in individuals with obesity, and 27%-92% in patients with hyperlipidemia[24,25]. Donati *et al*[26] showed that the prevalence of NAFLD in the patients with hypertension but without obesity or T2DM was 2 to 3-times higher than that in the general population. Assy *et al*[25] showed that up to 50% of the patients with fatty liver were dyslipidemic, and that this dysmetabolic condition was chiefly characterized by high serum TG levels, which itself is an important risk factor for cardiovascular disease.

NAFLD is closely related to incidence of cardiovascular disease. In fact, the most common causes of death in patients with NAFLD are atherosclerotic cardiovascular disease and hepatic cirrhosis[27]. Therefore, clinicians should not only consider central obesity, type 2 diabetes, dyslipidemia and hypertension as risk factors for NAFLD, but also pay more attention to them as high risk factors for cardiovascular, kidney and liver diseases. A key strategy for clinical treatment of NAFLD is to reduce the above risk factors, and this can be accomplished by applying the existing knowledge to generate effective public health policies for the prevention of this disease.

In summary, a high prevalence of fatty liver was discovered in physical examinees in Guangzhou. Some of the cases presented with mild or moderate increase in ALT or AST levels, but many had concomitant hyperlipidemia, hyperglycemia, hypertension, or hyperuricemia. Clinicians should pay attention to the intervention and modification of these risk factors. It is important to note, however, that the retrospective nature of this study limits the risk factors that were available for analysis; for example, data on waist circumference, body mass index, dietary habits, and alcohol consumption - all potential risk factors - were lacking. In addition, the single-site population may limit generalization of our results. More studies of larger Chinese populations are needed to gain more detailed information on fatty liver in the general population and to better guide clinical treatment.

ACKNOWLEDGMENTS

The authors are grateful to Shenzhen Jian An Pharmaceutical Company, Limited, for their support.

COMMENTS

Background

The incidence of fatty liver is relatively high and on the rise in urban populations of China; however, consultation rates are low due to a lack in adequate knowledge of fatty liver. To date, no study has examined the prevalence and clinical features of fatty liver in urban Chinese who receive routine physical examinations. Thus, this study was designed to retrospectively investigate the prevalence and presenting features of fatty liver in physical examinees of Guangzhou.

Research frontiers

Public health programs of screening, education, and treatment of fatty liver should start with employed urban Chinese, who are generally characterized as relatively well-educated, financially secure, and compliant. The results of this study may offer guidance for clinical treatment by analyzing the presenting features of fatty liver in this population.

Innovations and breakthroughs

This study is the first to assess the presenting features of fatty liver in urban Chinese who received routine physical examinations in Guangzhou. The disease was found to be closely associated with concomitant hyperlipidemia, hyperglycemia, hypertension, or hyperuricemia. The results from this study, which itself is part of a continuous clinical research effort for determining fatty liver diagnostic and prognostic factors, are applicable to the development of new programs for screening and education of fatty liver targeting urban Chinese.

Applications

This study was undertaken mainly for practical purposes, *i.e.*, to raise public awareness of fatty liver and support performance of screening in the general population, which are expected to improve consultation rates and timely initiation of treatment for fatty liver.

Peer review

This study represents the first published investigation of fatty liver prevalence detected by routine physical examinations of individuals residing in the Huangpu District of Guangzhou; the results suggest a close association between fatty liver and dysmetabolic factors. This study was undertaken mainly for practical purposes, such as to raise public awareness of fatty liver and the benefits of screening the general population for this disease so that cases may be diagnosed and treated in a timely manner.

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P- Reviewer Mo H **S- Editor** Gou SX **L- Editor** A
E- Editor Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5340

World J Gastroenterol 2013 August 28; 19(32): 5340-5346
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Clonality analysis of neuroendocrine cells in gastric adenocarcinoma

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Supported by The Natural Science Foundation of Zhejiang Province, China, No. Y2110133; the Zhejiang Provincial Medical Science Research Foundation, No. 2010KYA060
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Received: March 24, 2013 Revised: June 13, 2013
Accepted: July 4, 2013
Published online: August 28, 2013

Abstract

AIM: To achieve a better understanding of the origination of neuroendocrine (NE) cells in gastric adenocarcinoma.

METHODS: In this study, 120 cases of gastric adenocarcinoma were obtained. First, frozen section-immunohistochemistrical samples were selected from a large quantity of neuroendocrine cells. Second, laser capture microdissection was used to get target cells from gastric adenocarcinoma and whole genome amplification was applied to get a large quantity of DNA for further study. Third, genome-wide microsatellite abnormalities [microsatellite instability (MSI), loss of heterozygosity (LOH)] and *p53* mutation were detected by polymerase chain reaction (PCR)-single-strand conformation polymer- phism-silver staining and PCR-sequencing in order to identify the clonality of NE cells.

RESULTS: The total incidence rate of MSI was 27.4%, while LOH was 17.9%. Ten cases had a highest concordance for the two types of cells. The other samples had similar microsatellite changes, except for cases 7 and 10. Concordant *p53* mutations exhibited in sample 4, 14, 21 and 27, and there were different mutations between two kinds of cells in case 7. In case 17, mutation took place only in adenocarcinoma cells. *p53* mutation was closely related with degree of differentiation, tumor-node-metastasis stage, vessel invasion and lymph node metastasis. In brief, NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30). In the other three cases, different MSI, LOH or *p53* mutation occurred.

CONCLUSION: NE and the gastric adenocarcinoma cells may mainly derive from the same stem cells, but the remaining cases showing different origin needs further investigation.

Key words: Neuroendocrine differentiation; Clonal analysis; Gastric adenocarcinoma; Neuroendocrine cells

Core tip: There have been only a few studies of neuroendocrine differentiation (NED) in gastric adenocarcinoma. Therefore, we studied the clonality of neuroendocrine (NE) cells in gastric adenocarcinoma using laser capture microdissection, microsatellite instability (MSI), loss of heterozygosity (LOH) and *p53* mutation to evaluate the clonality of NED. NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30), they may originate from the same stem cells, but the remaining three cases showed different origins, which warrants further research.

Wang LL, Yao GY, Zhao ZS, Wei XL, Xu RJ. Clonality analysis of neuroendocrine cells in gastric adenocarcinoma. *World J Gastroenterol* 2013; 19(32): 5340-5346 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5340.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5340

INTRODUCTION

Although the worldwide incidence and mortality of gastric cancer have been declining steadily, it remains one of the most common cancers and the leading cause of cancer death worldwide[1]. Previous studies have reported that mixed glandular-neuroendocrine (NE) tumors that arise from the gastrointestinal tract, such as the stomach and colon, normally contain both glandular and endocrine cells[2,3]. These studies have suggested that mixed tumors occur as a consequence of multidirectional differentiation of glandular of endocrine stem cells that are derived from the endoderm. However, it remains unclear whether the glandular and endocrine cells expand from two distinct precursors, or arise from a single progenitor cell.

Microsatellite instability (MSI) is a form of genetic instability that is characterized by new alleles that are not present in the normal genotype. This type of mutation occurs in various human carcinomas[4], and is believed to be caused by altered DNA mismatch repair genes. Several genetic alterations have been shown to play a significant role in tumourigenesis. The most frequently observed molecular changes occur in the *p53* gene[5]. There is now enough evidence to suggest that the functional inactivation of the *p53* gene through allelic loss and point mutation plays an important role[6]. The *p53* gene encodes a protein that is involved in control of the cell cycle and acts as a negative regulator in the cell response to damaged DNA. The most widely used molecular approach is single-strand conformation polymorphism (SSCP) analysis of DNA fragments amplified by the polymerase chain reaction (PCR), with subsequent sequence analysis. Functional alteration of p53 protein can occur through several mechanisms: point mutations, deletions, rearrangements in the *p53* gene, binding with viral proteins, binding with cellular proteins, and oligomerization[7]. Wild-type p53 protein has a very short half-life, whereas mutated p53 is stable and can accumulate at high concentrations in the nuclei of tumor cells. As a consequence, immunohistochemical staining with specific antibodies can be used to detect mutant p53 protein.

To achieve a better understanding of the origination of NE cells in gastric adenocarcinoma, and provide a clear method of evaluation to clinicians, we performed a prospective study on neuroendocrine differentiation in gastric adenocarcinoma by analyzing MSI, loss of heterozygosity (LOH) and *p53* mutation.

MATERIALS AND METHODS

Frozen section immunohistochemistry

In this study, 120 cases of gastric adenocarcinomas and corresponding non-neoplastic gastric mucosal tissues were obtained from the People's Hospital of Zhejiang Province, China. The tumors were staged according to the tumor-node-metastasis (TNM) classification and were graded according to the World Health Organization classification. Immunohistochemistry was carried out using the primary antibody against NE marker (chromogranin A, polyclonal, 1:100; Maixin, China). In brief, the tissue sections were incubated in methanol for 5 min. After washing with phosphate-buffered saline (PBS), the sections were incubated in 7.5% hydrogen peroxide for 5 min, followed by further washing with PBS. The sections were then incubated with primary antibodies in the case of chromogranin A at 4 ℃ overnight. Then these sections were detected using Two-Step Immunohistochemical Detection Reagent (ZSGB-BIO, Beijing, China). Frozen section immunohistochemistry samples were selected from a large quantity of NE cells. The study was approved by the Ethics Committee for Human Study in our institution.

Laser-capture microdissection

Laser-capture microdissection (LCM) was performed with the use of an Arcturus PixCell II microscope (Arcturus Engineering, Mountain View, CA, United States) to obtain cells from gastric adenocarcinoma. The technology for melting heat of infrared rays was used to melt the polymeride under microscope, followed by molecular biology analysis. Open the instrument, put the complete slice on the objective table, the cell image was exhibited on the computer screen through the microscope. If the cellular morphology was normal, with satisfactory staining, under 10 × 20 lens according to the following conditions: power, 65 mV; duration, 15.5 s; and spots size, 7.5 μm, we attached the transparent Elvax® ethylene vinyl acetate hot plastic film hat by the driving arm to lay aside precisely above the tissue slice. The target cell or the cell group was obtained through the control handle to the slice migration located at the field of vision centre. Press the button according to the target region's size, and the focusing infrared laser beam carries on the capture. When the laser beam launch ended, move the mechanical arm from the slice to emigrate the cover and the thin film, move the hat into 0.5 mL Eppendorf centrifuge tube (add the Micro-kit extraction reagent box extraction buffer solution beforehand), and proceed with the DNA extraction. A 7.5-mm-diameter laser beam was used to procure NE cells and a 15- or 30-mm-diameter beam for adenocarcinoma cells. LCM cells were pooled from multiple caps, which were stored at -20 ℃ until dissection was complete. Approximately 15000 laser hits to each specimen gave the necessary cell yield after transfer. LCM was performed with capture of 500 NE cells and thousands of adenocarcinoma cells from each sample. NE and adenocarcinoma cell populations were stored separately. Cell samples were frozen immediately at -20 ℃, and were sent on the same day, on caps frozen on dry ice, for DNA extraction and subsequent genetic analysis.

DNA extraction and whole-genome amplification

DNA extraction from the captured cells and whole

Table 1 Primer sequences for the analysis of microsatellite instability and *p53* mutations at exons 5-8

Microsatellite	Sequences
D1S104	ATCCTGCCCTTATGGAGTGCCCCAC
	TCCTCTGTCATTGTA
D2S119	CTTGGGGAACAGAGGTCATTGAGA
	ATCCCTCAATTTCTTTGGA
D2S123	AAACAGGATGCCTGCCTTTAGGACT
	TTCCACCTATGGGAC
D3S1766	ACCACATGAGCCAATTCTGTACCCA
	ATTATGGTGTTGTTACC
D3S2427	CTCCTCGTCACTGCAGTCTTCTGCCT
	CATCTGTTCAGGAT
D4S174	AAGAACCATGCGATACGACTCATT
	CCTAGATGGGTAAAGC
D4S402	CTTACTGTGTTGCCCAAGGTAGCTC
	TATGATTCATTTCAAGTTTG
D5S107	GATCCACTTTAACCCAAATACGGC
	ATCAACTTGAACAGCAT
D5S346	ACTCACTCTAGTGATAAATCGGGA
	GCAGATAAGACAGTATTACTAGTT
D5S409	GGGATGAAGTGTGGATAAACTAGG
	ATGGCAGTGCTCTTAG
D7S1805	CCTGCTTTGGCTTACCTGTACCCAC
	TTCTCTGCTATTACATAT
D9S157	AGCAAGGCAAGCCACATTTCTGGG
	GATGCCCAGATAACTATATC
D10S469	CAACAAGTGTGAGAGTCCATATGTT
	CTGTCTCTCCACAGT
AFMA086WG9	ATGTACGGTTCATTGACTTGACTGA
	CTACAAATGGGCA
D11S861	CTGAAACCAAGTGAAAAGGAGAA
	AGCTCCATTGTCTTCTGGC
D12S1899	TTCTTCCTTTCTCTTTCTCTCTTCCGC
	ACAAGTGACACATGGTCC
D16S398	CTTGCTCTTTCTAAACTCCAGAAAC
	CAAGTGGGTTAGGTC
D16S496	GAAAGGCTACTTCATAGATGGCAA
	TATAAGCCACTGCGCCCAT
D16S534	CAACAAAGCAAGACCCTGTCCATC
	TGCGGTTCTTTCCTC
D16S265	AGCTCTCTGAGTCCTCTGTGCGGAA
	GCATGGTGTCTCTCG
D16S752	AATTGACGGTATATCTATCTGTCTG
	GATTGGAGGAGGGTGATTCT
D17S250	GGAAGAATCAAATAGACAATGCTG
	GCCATATATATATTTAAACC
D17S796	CAATGGAACCAAATGTGGTCAGTC
	CGATAATGCCAGGATG
D19S416	CCTGTCCCAGAGAGACCCTAAAGA
	GAGTGTGCCATTTGCT
BAT 25	GTTTCGCCTCCAAGAATGTAAGTGT
	TTCTGCATTTTAACTATGGCTC
BAT 26	TGACTACTTTTGACTTCAGCCAACC
	ATTCAACATTTTAACCC
Exon 5	GACTTTCAACTCTGTCTCCTCTGGG
	GACCCTGGGCAAC
Exon 6	GAGACGACAGGGCTGGGTCCACTG
	ACAACCACCCTT
Exon 7	GTGTTGTCTCCTAGGTTGGCAAGTG
	GCTCCTGACCTGGAG
Exon 8	CCTTACTGCCTCTTGCTTTGAATCTG
	AGGCATAACTGC

genome amplification (WGA) were performed using DNA Micro-kit and DNA Repli-g Midi kit (QIAGEN, Germany) to obtain a large quantity of DNA. The brief processes were as follows: 15 μL buffer ATL (provided in kit) was added to a 0.5-mL microcentrifuge tube that contained the laser-microdissected cells; 10 μL proteinase K was added and mixed by pulse-vortexing for 15 s; the 0.5-mL tube was then placed in a thermomixer or heated orbital incubator, and incubated at 56 ℃ for 3 h, with occasional agitation; 25 μL buffer ATL was added with 50 μL buffer AL, and mixed well by pulse-vortexing for 15 s; 50 μL ethanol (96%-100%) was added and mixed thoroughly by pulse-vortexing for 15 s, incubated for 5 min at room temperature. Then, the entire lysate was carefully transferred to the QIAamp MinElute Column, centrifuged at 8000 *g* for 1 min and placed in a clean 2-mL collection tube; 500 μL buffer AW1 and AW2 (provided in kit) were added, respectively, and centrifuged at 8000 *g* for 1 min, followed by a full speed centrifugation at 14000 *g* for 3 min to dry the membrane completely. The QIAamp MinElute Column was placed in a clean 1.5-mL microcentrifuge tube and 20-30 μL buffer AE was added to the centre of the membrane, incubated at room temperature for 1 min, and finally centrifuged at 14000 *g* for 1 min. The DNA was denatured by adding denaturation buffer and stopped by adding of neutralization buffer that contained DNA polymerase. The isothermal amplification reaction proceeded for at least 8 h at 30 ℃. The method was used based on a technology that carries out isothermal genome amplification utilising a unique processive DNA polymerase, which could replicate up to 100 kb without dissociating from the genomic DNA template. The DNA polymerase had a 3'-5' exonuclease proofreading activity to maintain a high fidelity during replication, and was used with exonuclease-resistant primers to achieve a high yield of DNA product. The final processes were: TE buffer and denaturation solution were added, mixed well and incubated at room temperature for 3 min; neutralization buffer was added, mixed, followed by adding REPLI-g master mix, and incubated for 8-16 h at 30 ℃; and REPLI-g Midi DNA polymerase was inactivated by heating the sample at 65 ℃ for 3 min.

Analysis of MSI, loss of heterozygosity and p53 mutation

We chose 26 microsatellite markers with genome-wide scope for MSI analysis, and chose p53 exons 5-8 for *p53* mutation analysis. The primers for these analyses are listed in Table 1.

Genome-wide microsatellite abnormalities (MSI and LOH) and *p53* mutation were detected by PCR-SSCP silver staining and PCR sequencing to identify the clonality of NE cells. To evaluate microsatellite alterations, extra shadow bands above and below each intense principal allelic band were often visualized in microsatellite studies, and the most intense bands were considered the real alleles.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, Chicago, IL, United States). Survival data were analysed using the χ^2 test, Spearman rank correlation analysis, and Kaplan-Meier analysis, and

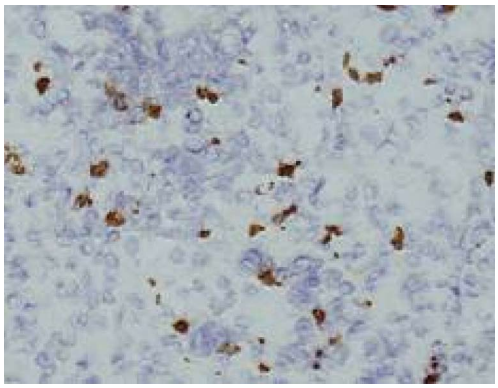

Figure 1 Chromogranin A expression in gastric cancer (× 100).

a survival curve was drawn. Differences were analysed using the log rank test and $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry and LCM

Thirty samples from a total of 120 that contained a large number of NE cells were selected for LCM. About 500 NE cells were precisely captured from each sample (Figures 1 and 2).

Microsatellite analysis and p53 mutation

The total incidence rate of MSI was 27.4%, and LOH rate was 17.9%. The rates in gastric adenocarcinoma cells and NE cells were similar. There was no significant relationship between the MSI or LOH rate and clinicopathological characteristics. According to the coincidence of microsatellite changes, cases 2, 3, 5, 6, 11, 12, 18, 24, 27 and 30 had a highest concordance for the two types of cells. The other samples had similar microsatellite changes, except for cases 7 and 10 (Figure 3).

Most mutations of the *p53* gene were detected in exons 7 and 8. Concordant mutations were observed in cases 4, 14, 21 and 27, and there were different mutations in the two types of cells (*e.g.*, NE and gastric adenocarcinoma cells) in case 7. In case 17, the mutation was seen only in the adenocarcinoma cells not in the NE cells. *p53* mutation occurred six times in adenocarcinoma cells (20.0%) and five times in NE cells (16.7%). Clinicopathological analysis further showed that *p53* mutations were well associated with poor differentiation and TNM stages Ⅲ or Ⅳ tumors, the mutations were also linked to blood vessel invasion and lymph node metastasis (Table 2).

DISCUSSION

Our previous studies have demonstrated that NED occurred in 41.5% of colon cancers, 39.6% of gastric cancers, 38.1% of prostate cancers, 21% of breast cancers and 17.9% of pancreatic cancers, and NE in gastric adenocarcinoma was more frequently observed in poorly differentiated cancers than in well-differentiated tumors[8],

Figure 2 Images shown before and after laser-capture microdissection (× 200). A: Before laser-capture microdissection (LCM); B: After LCM.

which was different from other studies that showed that NE was associated with well-differentiated tumors[9,10]. However, it is not clear whether NE is derived from embryogenesis, histogenesis, or genetic changes that are associated with tumor etiology. It has been shown that NED occurs in adenocarcinoma of the prostate, gastrointestinal tract and lungs. These NE cells synthesize and excrete neuropeptides or amines hormones, leading to an increase of plasma hormone levels[11-13]. Hirano *et al*[14] found that the prognosis for gastric adenocarcinoma with choriocarcinoma and neuroendocrine cell carcinoma is exceedingly poor. Whereas, the biological functions of NED for the development or prognosis of gastric adenocarcinoma are largely unknown. We thus employed LCM to capture NE cells, distinguished from the gastric adenocarcinoma cells, and utilized molecular and genetic approaches to study the origin of NE cells and their association with gastric cancer biology. We found that the NE cells and gastric adenocarcinoma cells shared similar MSI, LOH and *p53* mutation, meaning both cell lines may be derived from same stem cells.

It has been well known that the NE cells are derived from multipotent stem cells. NED is initiated by hormonal change, microenvironmental change, and genomic instability. Some subdued genomic codes are randomly depressed and selectively activated by more than two regulatory genes during RNA translation, and as a result, multipotent stem cells generate differentiation or multidifferentiation[15]. Despite the apparently different morphological representation of NE cells in the tumor

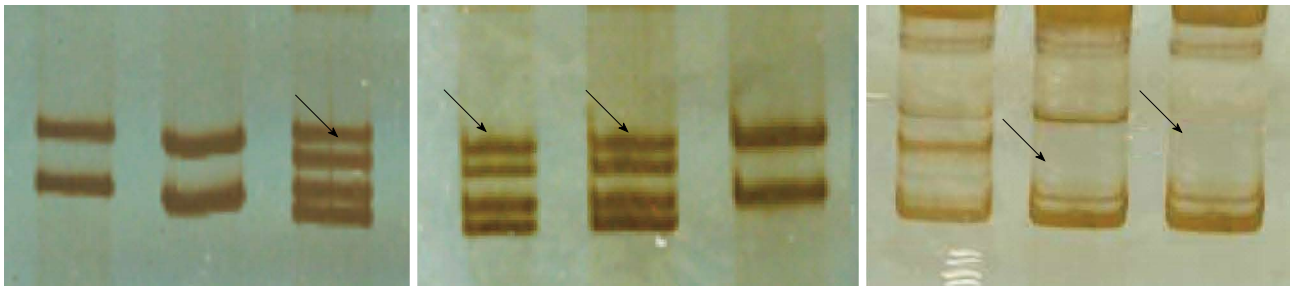

Figure 3 Examples of microsatellite instability and loss of heterozygosity in gastric cancer examined by single-strand conformation polymorphism analysis. NE: Neuroendocrine; T: Tumor samples; N: Normal tissue controls; MSI: Microsatellite instability; LOH: Loss of heterozygosity. Arrows show the increasing and missing of alleles.

Table 2 Concordance of *p53* mutation in gastric cancer and neuroendocrine cells

Case	Cell	Exon	Codon	Mutation	Amino acid	Differentiation	TNM	Metastasis
4	Cancer	8	273	GC→AT	Arg→Cys	Poor	Ⅳ	+
	NE	8	273	GC→AT	Arg→Cys			
7	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅳ	+
	NE	8	287	GC→AT	Glu→Lys			
14	Cancer	8	287	GC→AT	Glu→Lys	Poor	Ⅰ B	+
	NE	8	287	GC→AT	Glu→Lys			
21	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅳ	+
	NE	7	244	GC→AT	Gly→Ser			
27	Cancer	8	282	GC→AT	Arg→Arg	Moderate	Ⅲ	+
	NE	8	282	GC→AT	Arg→Arg			
17	Cancer	7	244	GC→AT	Gly→Ser	Poor	Ⅲ	+

NE: Neuroendocrine; TNM: Tumour-node-metastasis.

mass, it is largely unknown whether these cells have chromosomal or genetic alterations. Moreover, it is not clear whether NE cells are present as tumor or stromal components. NE cells from gastric adenocarcinoma were harvested by LCM, which ensured cell purity. Whole genome amplification (WGA) was then employed to compare genomic characteristics of NE cells with adenocarcinoma cells, for the identification of the clonality of the former. The development and prognosis of gastric cancer involves a number of genetic and epigenetic abnormalities[16]. MSI is thought to be an important molecular phenotype in gastric cancer[17]. In gastric cancer, the loss of genomic stability represents a key molecular step that occurs early in carcinogenesis, and creates a permissive environment for the accumulation of genetic and epigenetic alterations in tumor suppresser genes and oncogenes. It is widely accepted that gastric cancer can follow at least two major genomic instability pathways: MSI and chromosome instability[18]. LOH and MSI have strong sensitivity but poor specificity, whereas gene mutation has strong specificity but poor sensitivity. The appropriate combination of the two methods can give more precise results. Huang *et al*[19] have demonstrated whether different components of combined tumors contain the same or different genetic alterations, thus providing evidence for their clonality. As a result, he has suggested that, in the majority of combined tumors, cells with different phenotypes share similar genotypes and might arise from a single precursor cell. Only in a minority of these tumors are different areas derived from different precursor cells. Our study suggested that concordant microsatellite changes occurred in two types of cells in cases 2, 3, 5, 6, 11, 12, 18, 24, 27 and 30; different microsatellite changes in cases 7 and 10; and in the remaining 18 cases, there were no significant differences in microsatellite changes in the two types of cells. There was no correlation between MSI and degree of differentiation in gastric cancer. Semba *et al*[20] have suggested that MSI appears at a high frequency in well-differentiated adenocarcinoma, but others have come to the opposite conclusion[21].

Wild-type *p53* acts as an anti-oncogene in normal tissues, which is important in DNA repair and cell cycle regulation. Tumourigenesis is closely associated with *p53* mutation or loss of function[22]. Genetic changes (such as gain or loss of chromosomal segments, or gene mutation) in allelic genes are induced by unbalanced mitosis during stem cell differentiation. These genetic changes could be used for analysis of cell clonality. They can be detected by microsatellite changes (including LOH and MSI), gene mutation, and comparative genomic hybridisation. The functional inactivation of *p53* gene through allelic loss and point mutation plays an important part in the development of gastric cancer. We can detect mutant p53 protein by immunohistochemical staining with specific antibodies[23,24]. Nishikura *et al*[25] have suggested that NE carcinoma is composed of precursor NE cells that are

generated from adenocarcinoma, and p53 promotes this process. While studying gastrointestinal carcinomas, Eren has discovered that *p53* mutation might be associated with NED of adenocarcinoma[26]. The rate of p53 positivity in gastric carcinoma with NED was clearly higher than that in gastric carcinoma without NED. Our study showed that the rate of *p53* mutation in gastric adenocarcinoma cells was 20%, and it was 16.7% in NE cells. In cases 4, 14, 21 and 27, concordant mutations were seen in exons 7 and 8 in the two types of cells; in case 7, different *p53* mutations were observed; and in case 17, *p53* mutation was only seen in adenocarcinoma cells and not in the NE cells. The concordance rate of *p53* mutation in the two types of cells was 66.7%. Based on the similar microsatellite changes and *p53* mutations in both NE cells and adenocarcinoma cells in the 27 of 30 cases, we claimed that the NE and adenocarcinoma cells probably were derived from the same stem cells. Our results provided more evidence to support that the multipotent stem cells could differentiate to NE and adenocarcinoma cells. Whether NE cells can act as parenchyma of carcinoma and secrete hormones to promote carcinoma needs further investigation. We also found that 3 cases showed different MSI, LOH and *p53* mutation pattern, suggesting that the NE and gastric adenocarcinoma cells were derived from different stem cells. Further study on the underlying mechanisms is needed.

ACKNOWLEDGMENTS

We would like to thank Professor Wancai Yang (University of Illinois at Chicago, Chicago, United States) for critical discussion and English language revision.

COMMENTS

Background

Neuroendocrine differentiation (NED) is a common phenomenon in adenocarcinomas, but there have been only a few studies of NED in gastric adenocarcinoma. It remains unclear whether the glandular and endocrine cells expand from two distinct precursors, or arise from a single progenitor cell.

Research frontiers

Authors used laser capture microdissection, microsatellite instability (MSI), loss of heterozygosity (LOH) and *p53* mutation to evaluate the clonality of NED.

Innovations and breakthroughs

Authors studied the clonality of neuroendocrine (NE) cells in gastric adenocarcinoma using laser capture microdissection, MSI, LOH and *p53* mutation to evaluate the clonality of NED. NE and adenocarcinoma cells showed the same MSI, LOH or *p53* mutation in most cases (27/30), which may originate from the same stem cells. In the other three cases, different MSI, LOH or *p53* mutation occurred.

Applications

The article helps to achieve a better understanding of the origination of NE cells in gastric adenocarcinoma, and provide a clear method of evaluation to clinicians.

Terminology

Laser-capture microdissection (LCM): LCM was performed to obtain cells from gastric adenocarcinoma. The technology makes use of the melting heat of infrared rays to melt the polymeride under microscope, followed by molecular biology analysis.

Peer review

The authors discuss the available information on LOH and MSI in view of their findings and published reports. They presented from their and other groups the findings on p53 in gastric cancer and argued that NE and adenocarcinoma cell likely derive from the same stem cell in the majority of the tested tumors. In brief, this is an interesting study that is thoroughly performed and interpreted.

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P- Reviewers Rodriguez DC, Tarnawski AS, Zoller M
S- Editor Gou SX **L- Editor** Ma JY **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5347

World J Gastroenterol 2013 August 28; 19(32): 5347-5356
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Efficacy profiles for different concentrations of *Lactobacillus acidophilus* in experimental colitis

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Received: March 13, 2013 Revised: June 3, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

AIM: To determine the efficacy profiles of different concentrations of *Lactobacillus acidophilus* (*L. acidophilus*) for treating colitis using an experimental murine model.

METHODS: Colitis was established in 64 BALB/c mice by adding 5% dextran sodium sulfate (DSS) to the drinking water and allowing *ad libitum* access for 7 d. The mice were then randomly divided into the following control and experimental model groups (n = 8 each; day 0): untreated model control; negative-treatment model control (administered gavage of 1 mL/10 g normal saline); experimental-treatment models C4-C8 (administered gavage of 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/10 g *L. acidophilus*, respectively); positive-treatment model control (administration of the anti-inflammatory agent prednisone acetate at 45 μg/10 g). Eight mice given regular water (no DSS) and no subsequent treatments served as the normal control group. Body weight, fecal traits, and presence of fecal occult blood were assessed daily. All animals were sacrificed on post-treatment day 7 to measure colonic length, perform histological scoring, and quantify the major bacteria in the proximal and distal colon. Intergroup differences were determined by one-way ANOVA and post-hoc Student-Newman-Keuls comparison.

RESULTS: All treatments (*L. acidophilus* and prednisone acetate) protected against colitis-induced weight loss ($P < 0.05$ *vs* model and normal control groups). The extent of colitis-induced colonic shortening was significantly reduced by all treatments (prednisone acetate > C4 > C5 > C7 > C8 > C6; $P < 0.05$ *vs* untreated model group), and the C6 group showed colonic length similar to that of the normal control group ($P > 0.05$). The C6 group also had the lowest disease activity index scores among the model groups. The bacterial profiles in the proximal colon were similar between all of the experimental-treatment model groups (all $P > 0.05$). In contrast, the bacterial profile in the distal colon of the C6 group showed the distinctive features ($P < 0.05$ *vs* all other experimental-treatment model groups) of *Lactobacillus* sp. and *Bifidobacterium* sp. being the most abundant bacteria and *Staphylococcus aureus* being the least abundant bacteria.

CONCLUSION: The most therapeutically efficacious concentration of *L. acidophilus* (10^6 CFU/10 g) may exert its effects by modulating the bacterial profile in the distal colon.

Key words: *Lactobacillus acidophilus*; Bifidobacterium; Colonic flora; Therapeutic dose; Experimental colitis; Efficacy profile

Core tip: Efficacies of the current treatments for ulcerative colitis (UC) are limited by procedure-related complications, poor patient compliance, and high re-

lapse rates. Administration of supplemental probiotics represents a promising new therapy of UC. Since UC pathogenic sites mainly involve the rectum and colon and UC patients show differential composition profiles of intestinal bacteria, this study was designed to evaluate the therapeutic efficacies of various concentrations for the standard probiotic, *Lactobacillus acidophilus*, using a well-known murine model of experimental colitis to examine the changes in colitis symptoms and the corresponding effects on the bacterial flora in the distal and proximal colon.

Chen LL, Zou YY, Lu FG, Li FJ, Lian GH. Efficacy profiles for different concentrations of *Lactobacillus acidophilus* in experimental colitis. *World J Gastroenterol* 2013; 19(32): 5347-5356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5347.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5347

INTRODUCTION

Ulcerative colitis (UC), a non-specific chronic inflammatory bowel disease, has emerged as a significant human health burden in Western countries and its prevalence is rising worldwide. As such, extensive research efforts have focused on determining its pathogenic mechanisms and developing efficacious therapies. While these studies have helped to define the clinical course of UC, identification of a safe and effective treatment has remained elusive. The primary drug therapies currently in use, including the anti-infective salazosulfamide, anti-inflammatory glucocorticoids and immunosuppressant probiotics, are limited by considerable side-effects, which lead to poor patient compliance and may contribute to the high relapse rates of UC[1].

Four fundamental components underlying UC pathogenesis have been identified, which represent likely sources for the yet undefined etiological factors: environment, microbiota, immune system, and genome[2,3]. A large number of experimental studies using animal models and clinical studies of human UC subjects have demonstrated that the intestinal microbiota, in particular, plays an important role in both UC onset and progression[4-7]. Moreover, sterile conditions (*i.e.*, germfree environments) have been shown to induce UC in mice and differential distributions of specific bacteria (*i.e.*, *Campylobacter* sp.) have been correlated with UC in adult humans[8,9]. Interactions between the microbiota and the immune system are well-described and recognized for their critical roles in normal physiological processes; accordingly, aberrant development and response of the immune system related to the microorganism environment in the gut, have been associated with UC[2,10].

The profile of normal human intestinal flora consists of about 30 genera of bacteria, representing hundreds of species and unknown thousands of strains. The most abundant species are anaerobic (including Bifidobacteria, Lactobacilli and Bacteroides), among which the *Lactobacillus* sp. appear to be the predominant flora, especially in the colon. Furthermore, quantitative analysis has indicated that many of these anaerobes are present at concentrations between 10^9-10^{12} CFU/mL. Many of these anaerobes, such as *Lactobacillus* sp. and *Bifidobacterium* sp., are characterized as probiotics, exerting beneficial effects on the human body. Indeed, detection of latent pathogenic species, such as *Clostridium* and *Staphylococcus*, is rare under normal physiological conditions[4].

Probiotics are considered a promising alternative therapy for UC. To date, Lactobacilli, Bifidobacteria, VSL#3 (a compound probiotic preparation composed of four Lactobacilli strains, three Bifidobacteria strains, and one *Streptococcus salivarius* strain), and *Escherichia coli* (*E. coli*) Nissle 1917 have been applied to UC subjects (both animal models and human cases) and shown to effectively resolve disease symptoms[11-14]. In addition, our laboratory showed that administration of *Lactobacillus acidophilus* (*L. acidophilus*) at the early stage is an effective therapy for UC but that administration of different probiotics does not provide the same efficacies[15,16]. These results suggest that the distinctive features of different bacteria, including their secretory functions and interactions with other bacteria and the host system, may have significant functional implications for their therapeutic efficacies in specific host tissues. Thus, while it is possible that introducing a large amount of a probiotic (or a mixture of probiotics) may beneficially impact the overall profile of intestinal bacteria, such an effort may also provide no benefit or be detrimental, possibly promoting latent pathogenicity, in specific intestinal regions.

Since UC pathogenic sites mainly involve the rectum and colon[3] and UC patients show differential composition profiles of intestinal bacteria[17,18], we hypothesize that development of probiotic therapy as an effective UC treatment modality will depend upon the particular probiotic's effects at a specific tissue site, dosage concentration, and relationship to other flora. Thus, the current study was designed to evaluate the efficacy profiles of different concentrations (10^4-10^8/10 g body weight) of the probiotic *L. acidophilus* in the proximal and distal colon of a well-established murine model of experimental colitis.

MATERIALS AND METHODS

Bacterial strain

L. acidophilus was isolated from a normal human intestinal tract SMC-S095 sample and sequence-identified by our laboratory. After culturing under anaerobic conditions with MRS medium for 24 h, the bacteria was collected, quantified by a spectrophotometer, and diluted with normal saline (NS) to 10^{10} CFU/mL.

Study design

Seventy-two female BALB/c mice (6-8-wk-old, 20.0 ± 2.0 g mean body weight) were purchased from Hunan Agricultural University and housed under standard con-

ditions (50% ± 10% humidity, 12 h light/dark cycle, *ad libitum* access to standard mouse chow). Colitis was established in 64 of the mice by adding 5% dextran sodium sulfate (DSS, MW 50000; Sigma Corp, St. Louis, MO) to the drinking water and allowing *ad libitum* access for 7 d. The mice were then randomly divided into the following control and experimental model groups (n = 8 each; day 0): untreated model control; negative-treatment model control (administered gavage of 1 mL/10 g normal saline); experimental-treatment models C4-C8 (administered gavage of 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/10 g *L. acidophilus*, respectively); positive-treatment model control (administration of the anti-inflammatory agent prednisone acetate at 45 μg/10 g). Eight mice given regular water (no DSS) and no subsequent treatments served as the normal control group.

Body weight, fecal traits, presence of fecal occult blood, and disease activity index (DAI) scores were assessed daily, as previously described[19]. On post-treatment day 7, all mice were sacrificed by ether anesthesia overdose. The resected colon was measured (length-wise). Colonic segments (0.5 cm) were obtained starting from 1 cm to the ileocecus to 1 cm to the anus and used for bacterial analysis (described below). The remaining colonic segments approximately 0.5 cm to the anus were fixed in neutral formalin, prepared as paraffin-embedded sections, stained with hematoxylin and eosin (HE), and subjected to histological analysis by light microscopy and the damage scoring procedure described by Dieleman *et al*[20].

Bacterial analysis of intestinal flora

Colonic segments were weighed, homogenized, serially diluted in NS, and used to inoculate nonselective and selective culture mediums. For each animal, 10 L of intestinal fluid (10^0-10^{-5} *L. acidophilus* concentration) was collected and also inoculated in corresponding media. After culturing, three isolates each of anaerobic bacteria (Lactobacilli, Bifidobacteria, and Bacteroides) and aerobic bacteria [*Staphylococcus aureus* (*S. aureus*), *E. coli*, and Enterococci], as well as a sample of total aerobic bacteria, were selected for further analysis.

The anaerobic bacteria were incubated in the BAC. III-IE anaerobic workstation (Shel Lab, Cornelius, OR) at 37 ℃ for 48-72 h using the appropriate medium (Lactobacilli, LBS medium; Bifidobacteria, BS medium; Bacteroides, BDS medium; total aerobic bacteria, ordinary nutrition agar medium). The aerobic bacteria were incubated in the BSG-4 biochemical incubator (WanTong Precision Instruments Co., Ltd, Wuhan, China) at 37 ℃ for 24-48 h using the appropriate medium (*S. aureus*, high-salt mannitol medium; *E. coli*, eosin-methylene blue medium; Enterococci, TTC sodium azide medium). The resultant bacterial colonies were counted and converted to CFU/g by the following formula: number of bacterial colonies × [(diluted liquid volume + sample weight)/ sample weight] × dilution multiple.

Bacteria identification

Preliminary identification was performed on colonies of different bacteria according to morphological and Gram staining characteristics detected by light microscopy.

Identification of anaerobic bacteria (Lactobacilli and Bifidobacteria): Using Bifidobacteria as an example, the bacterial colonies of various forms were obtained from the BS medium, cultured respectively in MRS liquid culture medium. Resultant colonies were analyzed by light microscopy to confirm normal homogeneous morphology of Bifidobacteria, and isolates were inoculated onto MRS solid culture medium and cultured in the anaerobic incubator at 37 ℃ for 24 h. The resultant colonies were rescreened. After several generations' of serial cloning, the bacterial colonies with normal homogeneous appearance and morphology were selected for bacterial identification *via* the API20 A test (bioMérieux Vitek, Inc., Hazelwood, MO), according to the manufacturer's instructions. Results were within the API20 Analytical Profile Index. The protocol for Lactobacillus identification was similar.

Identification of Enterococci: The serially purified bacteria isolated from TTC culture were verified as Enterococci according to results of catalase test and verified by Gram staining. A single colony was resuspended in 0.3 mL sterile water, inoculated on a sheep blood agar plate, and incubated in the biochemical incubator at 37 ℃ for 24 h. One of the resultant colonies was selected for testing with the API20 Strep test (bioMérieux Vitek, Inc.), according to the manufacturer's instructions. Results (at 4 and 24 h of culture) were within the API20 Analytical Profile Index.

Statistical analysis

All statistical analyses were carried out with the SPSS statistical software suite (version 16.0; SPSS Inc, Chicago, IL). Results are expressed as mean ± SD. Quantitative data were converted to logarithm values and tested for homogeneity of variance. If the variance was homogeneous, single-factor analysis of variance (one-way ANOVA) was used to analyze the differences between groups. Mean values with significant difference ($P < 0.05$) were subjected to post-hoc pairwise comparison by the Student-Newman-Keuls test.

RESULTS

Effects of L. acidophilus treatments on colitis-induced changes in the general condition, body weight, and colonic length of mice

General condition: Mice in the normal control group were alert and had sleek, healthy coats. Mice in the untreated model control and the negative-treatment model control groups were apathetic, inert, had horripilated, dry coats with blood staining around the perianal area, and showed an obviously leaner body configuration than the normal controls. All experimental-treatment model groups and the positive-treatment model group showed similar disruptions in alertness, motor function, and coat condition as their model control counterparts, but to a much lesser extent.

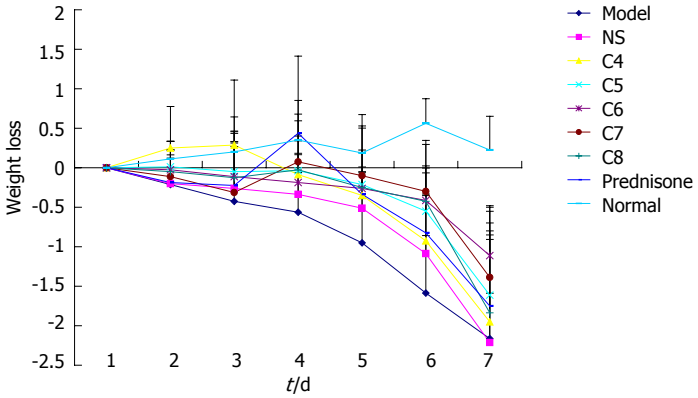

Figure 1 Effects of *Lactobacillus acidophilus* treatments on colitis-induced body weight loss. The *x*-axis shows the mean weight (mean ± SD) recorded for each group on each day of the 7-d study course. NS: Negative-treatment.

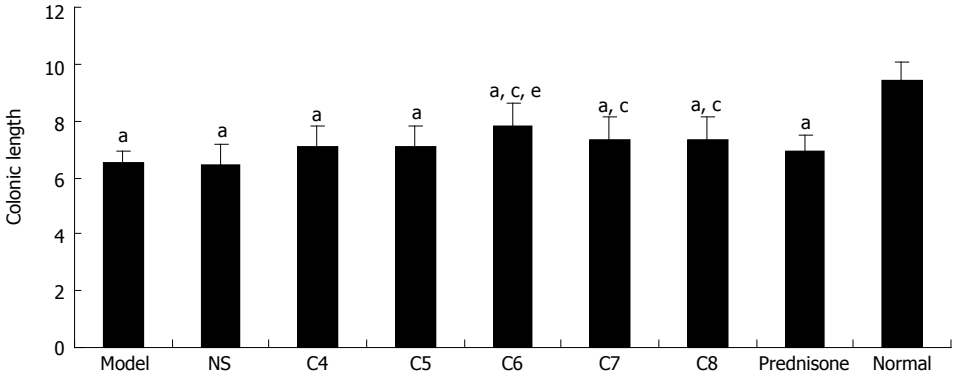

Figure 2 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in colonic length. $^{a}P < 0.05$ *vs* normal control group; $^{c}P < 0.05$ *vs* untreated model control group; $^{e}P < 0.05$ *vs* negative-treatment (NS) model control group.

Body weight: Mice in the normal control group experienced an increase in body weight over the duration of the study course; all mice were within normal weight range at sacrifice. In contrast, all other mice experienced a decrease in body weight over the study course. The greatest weight loss occurred in the untreated model control group, and the negative-treatment model control group showed only slightly less (and statistically similar; $P > 0.05$) weight loss. The body weight loss experienced by all experimental-treatment model groups and the positive-treatment model group showed trends of a more gradual decline over time than that of the untreated model control group. On the day of sacrifice, the extent of body weight loss among the model groups (Figure 1) showed the following hierarchical pattern: untreated model control = negative-treatment (NS) model control > C4 > C8 > positive-treatment (prednisone acetate) model control > C5 > C7 > C6.

Colonic length: All model groups had significantly shorter colonic lengths than the normal control group ($P < 0.05$), with the greatest extent of shortening observed in the untreated model control and negative-treatment model control groups. As shown in Figure 2, the following hierarchical pattern was observed for shortening degree among the treatment model groups: positive-treatment (prednisone acetate) model control > C4 > C5 > C7 > C8 > C6. Compared to the untreated model control group, the mean colonic lengths of C7, C8, and C6 were significantly longer ($P < 0.05$). Among those groups, the C6 group experienced the smallest degree of colonic shortening, and its mean colonic length was similar to that of the normal control group ($P > 0.05$).

Effects of L. acidophilus treatments on colitis-induced changes in DAI scores

When the mean DAI score of the normal control group was set to zero, the mean DAI score of all model groups showed a trend of significantly increasing values (indicating increasing detrimental pathology) over the study course. On the day of sacrifice, the untreated model control and the negative-treatment model control groups showed the highest (and similar) DAI scores. During the first four days after treatment, no significant differences were observed between the model groups (controls and experimentals). However, starting at day 5 post-treatment, the scores of the untreated model control and the negative-treatment model control groups markedly increased, indicating that the UC in these mice was aggravated. In addition, at this time, therapeutic effects begin to appear among the -treatment model groups. As shown in Figure 3A, while all model groups showed a sharp increase in mean DAI scores at day 7, the scores for the C4 and prednisone acetate treatment groups became significantly higher than the other experimental-treatment groups and statistically similar to those of the model control groups (both $P < 0.05$). As shown in Figure 3B, the experimental-treatment groups showed the following hierarchical

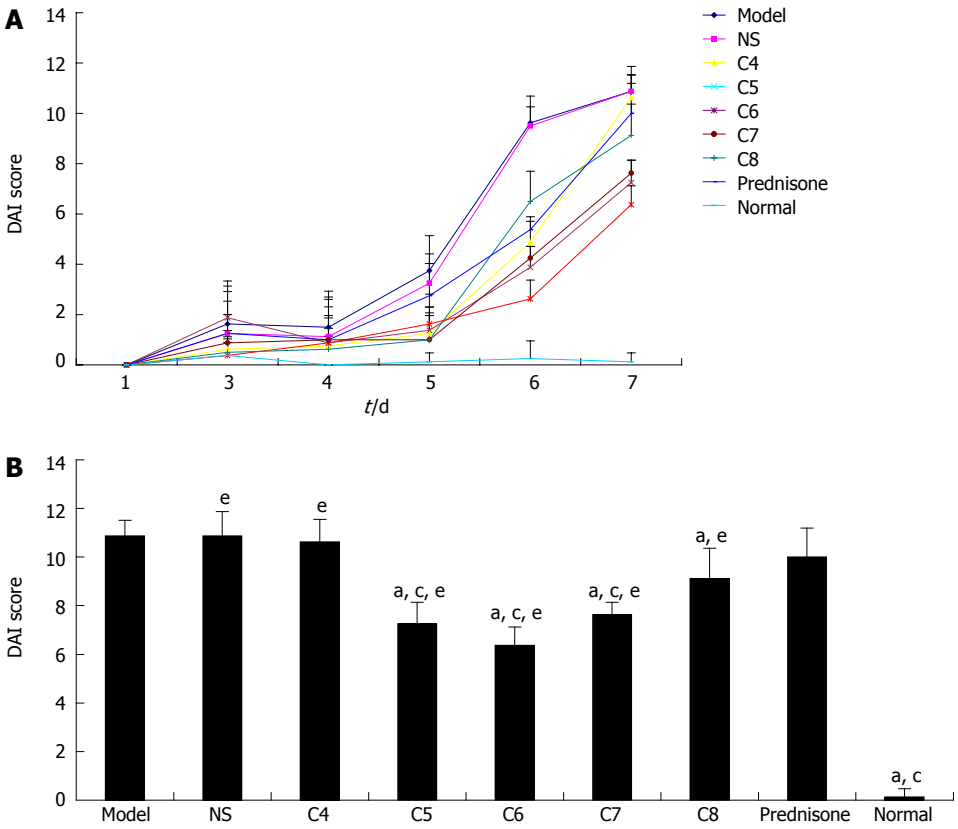

Figure 3 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in disease activity index scores. Comprehensive evaluation of body weight decrease, fecal properties, and fecal occult blood was carried out by the disease activity index (DAI) scoring system throughout the study course. A: The daily mean ± SD DAI score is plotted for each group; B: Mean ± SD DAI scores on day 7 post-treatment. [a]$P < 0.05$ *vs* untreated model control group; [c]$P < 0.05$ *vs* positive-treatment (prednisone acetate) model control group; [e]$P < 0.05$ *vs* normal control group. NS: Negative-treatment.

pattern of decreasing DAI scores on day 7: C8 > C7 > C5 > C6.

Effects of L. acidophilus treatments on colitis-induced changes in histopathological features of the intestinal tissues

Mice in the normal control group showed normal orderly arrangement of the cellular structure of rectal and colonic tissue glands, with no obvious perturbations in goblet cell number or mucosal integrity. In contrast, mice in the untreated model control and the negative-treatment model control groups showed multifocal and deep ulcers distributed throughout the entire colon; moreover, the extent of ulceration increased along with disease severity, as evidenced by recess depth, histological perturbations, mucosal erosion, bleeding, necrosis, partially or completely damaged epithelial cell structure, and inflammation extending towards the submucosa and serosa. Mice in the experimental-treatment model groups and the positive-treatment model group also showed mucosal defects similar to but less extensive than those in the model control groups; moreover, the extent of relief of the mucosal defects varied among the different treatment models. The C6 group, in particular had the most apparent relief of colitis-induced mucosal defects, showing partially incomplete glands, rare occurrences of mucosal erosion, bleeding and necrosis, and only a small quantity of inflammatory cell infiltration (Figure 4A, panels 1-9).

The histological scores of colon damage observed in the various groups at day 7 are shown in Figure 4B. When the mean damage score of the normal control group was set to zero, the mean damage scores of all model groups showed a trend of significantly increasing values over the study course. The untreated model control and the negative-treatment model control groups had the highest mean damage scores. Compared to the untreated model control group, the mean damage scores of all experimental-treatment groups and the positive-treatment control model group were significantly lower ($P < 0.05$). Comparisons among the treatment model groups revealed that the mean damage scores of C6 and C7 groups were significantly lower than that of the prednisone acetate group ($P < 0.05$), with the C6 group having the lowest score.

Effects of L. acidophilus treatments on colitis-induced changes in the proximal and distal colonic bacterial profiles

The bacterial profiles detected in the proximal colons of the control, model, and treatment model groups were similar, and none of the between-group differences reached statistical significance (Figure 5A). In contrast, the bacterial profiles detected in the distal colons showed distinct features between the various groups (Figure 5B).

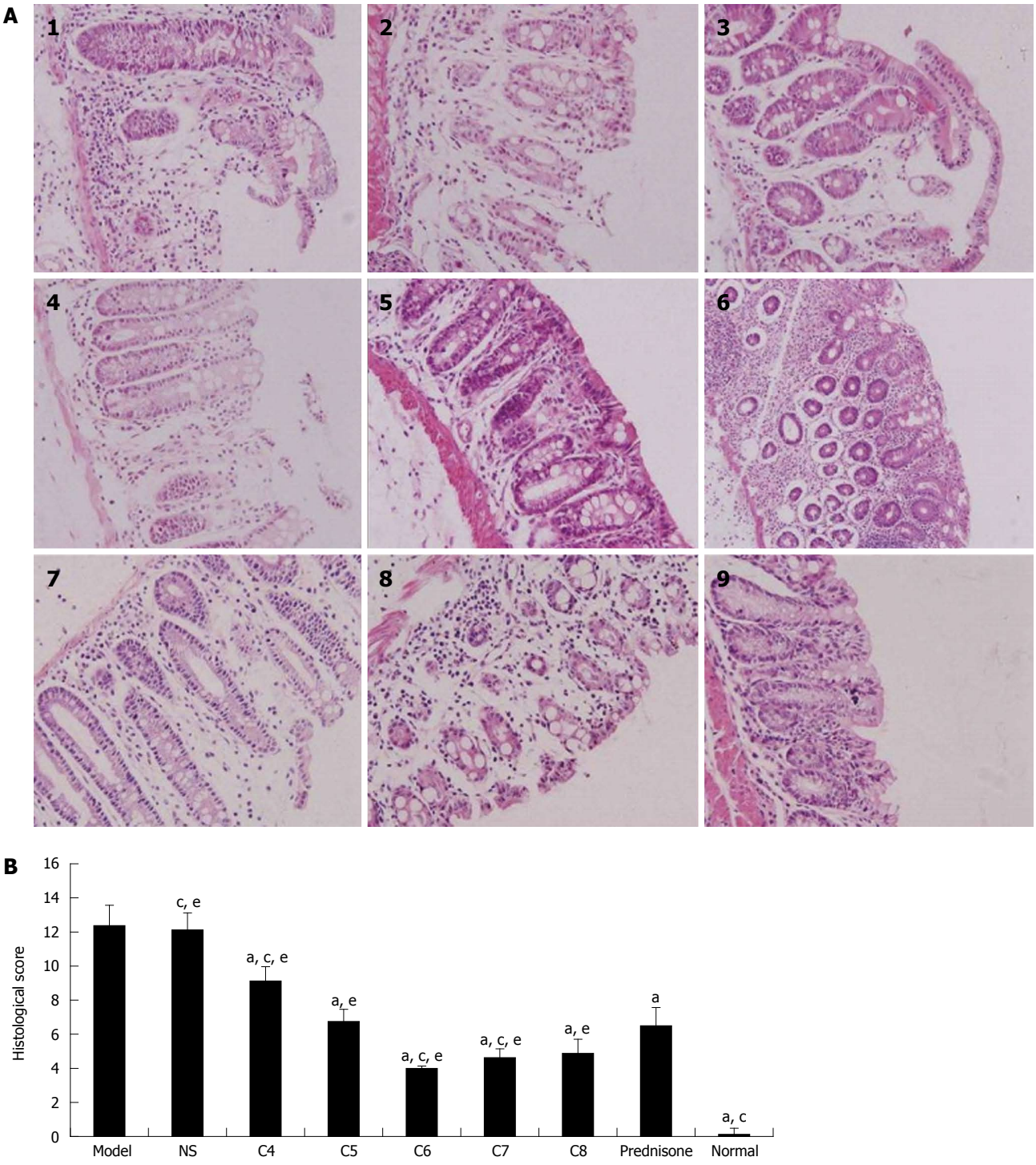

Figure 4 Effects of *Lactobacillus acidophilus* treatments on colitis-induced changes in intestinal histopathological features. A: Representative intestinal sections (HE, under light microscope, × 200) are shown. Panel 1: untreated model group, 2: negative-treatment (NS) model control group, 3-7: C4-C8 experimental treatment model groups respectively, 8: positive-treatment (prednisone acetate) model control group, and 9: normal control group; B: Average histopathological scores. $^{a}P < 0.05$ *vs* untreated model control group; $^{c}P < 0.05$ *vs* positive-treatment (prednisone acetate) model control group; $^{e}P < 0.05$ *vs* normal control group.

While the most abundant bacteria in all of the profiles were Lactobacilli, Bifidobacteria and Staphylococcus, the levels of each were different. For example, the levels of Lactobacilli among the untreated, negative-treatment, and positive-treatment model control groups were significantly higher than those in the normal control group ($P < 0.05$) but similar to one another ($P > 0.05$), while the experimental-treatment groups showed the highest levels ($P < 0.05$ *vs* the untreated model control group and the positive-treatment model control group), The levels of Bifidobacteria showed the same trends as the Lactobacilli levels. The levels of *S. aureus*, however, were similar between the untreated model control group, the negative-treatment model control group, the positive-treatment

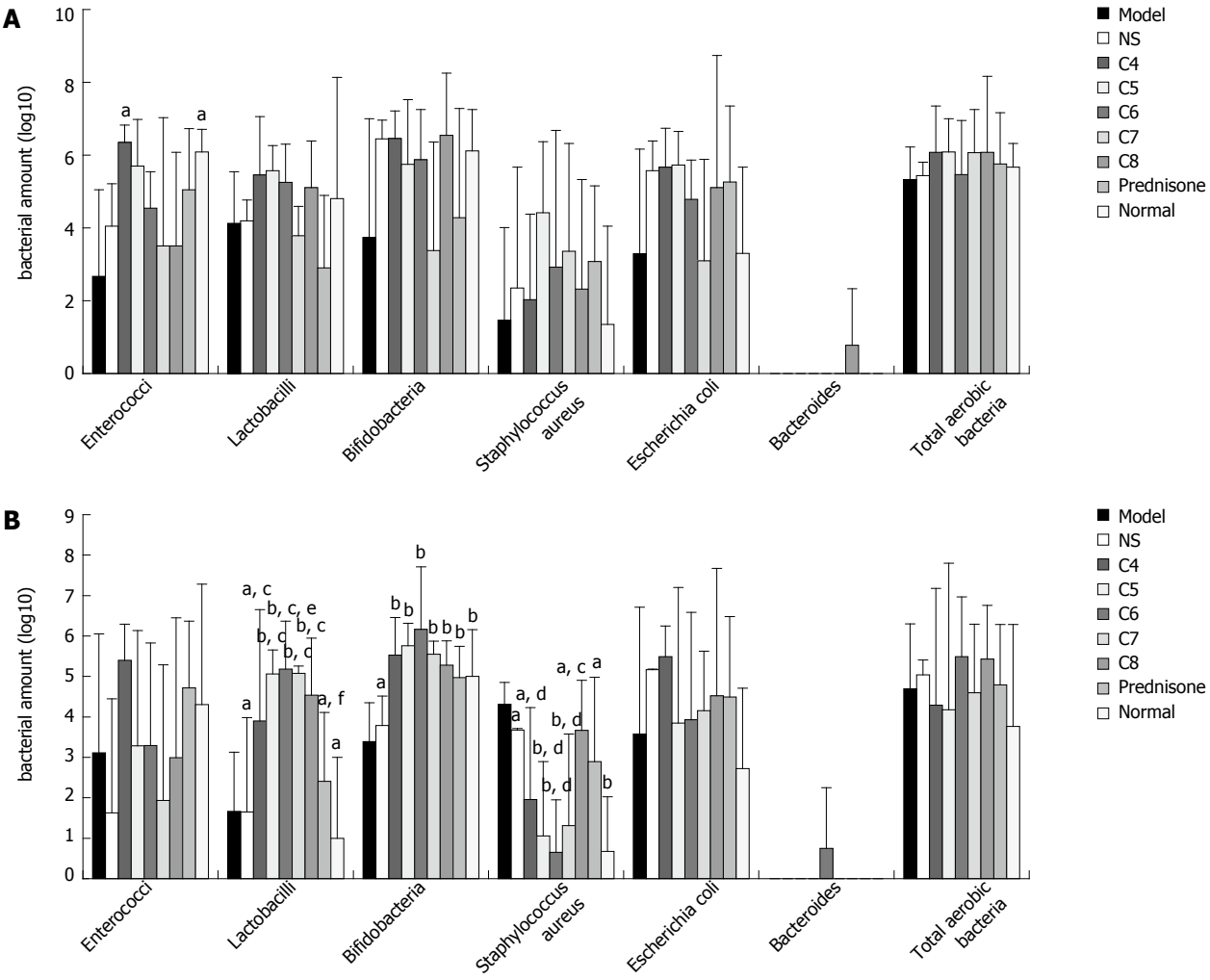

Figure 5 Bacterial profiles of the proximal (A) and distal (B) colon. Bacteria amount is expressed as the logarithm. [a]$P < 0.05$, [b]$P < 0.01$ *vs* untreated model control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* positive-treatment (prednisone acetate) model control group; [e]$P < 0.05$, [f]$P < 0.01$ *vs* normal control group. NS: Negative-treatment.

model control group, the C4 group, and the C8 group ($P > 0.05$), but all five were significantly higher than that in the normal control group ($P < 0.05$). The C6 group showed the lowest level of *S. aureus* among all the model groups ($P < 0.05$), and this amount was not significantly different from that in the normal control group ($P > 0.05$). No other bacteria detected showed significantly different levels between any of the groups.

DISCUSSION

To date, investigations of the potential correlations between bacterial profiles and UC have been carried out from the perspective of disease cause (etiological factors) and resolution (therapeutic modalities). Despite the focused efforts of many experimental and clinical studies, no particular bacterial genus or species, or combined panel of such, has been identified as a causative agent of UC onset. Profiling of the organic acids metabolized by bacteria that are present in stool samples has indirectly provided some insights into this issue, suggesting that UC is likely to be related to a panel of multiple bacteria, rather than a single species or phenotype[21]. Profiling of human intestinal flora has indicated that individuals with UC have significantly higher numbers of intestinal *Bacteroides* sp., *Streptococcus* sp., and facultative anaerobes, but significantly lower numbers of *Lactobacillius* sp. and *Bifidobacterium* sp. than their healthy counterparts[21-23]. These results are considered to have clinical implications in that they suggest administration of corresponding probiotics may restore the profile of enteric microorganisms to match that of a non-UC status.

Indeed, several studies to date have evaluated the therapeutic efficacy of probiotic administration using Lactobacilli[24,25], Bifidobacteria[11,26,27], *E. coli* Nissle 1917[16,28], or VSL#3[15,29-31]. The degrees to which these individual supplements successfully resolved the UC varied, which led to the hypothesis that administration of a combination of probiotics may provide more benefit to the patients. In order to determine the most efficacious composition, the overall profile of bacteria present at the mucus barrier in UC needs to first be determined[32]. Another important issue that needs to be elucidated is the activities and effects of the various probiotics on the normal intestinal flora; otherwise, a suboptimal dose of any particular probiotic may negatively impact the overall

efficacy of the treatment.

Gionchetti *et al*[30] demonstrated that high-dose VSL#3 helps to maintain the remission status achieved by surgery to treat mild pouchitis of UC; lower doses were not evaluated. An informal review of the research studies of probiotic treatment for UC published in the publicly accessible science and medical literature databases suggested that most common concentrations of probiotics used range between 10^6-10^9 CFU/mL; the lack of a standardized concentration precludes direct comparison of the results from these studies. In addition, we have found no published reports of comparative analyses to evaluate the differential effects of probiotics at varying concentrations.

L. acidophilus was chosen as the focus of the current study based upon previous results showing its therapeutic benefit for early-stage experimental colitis and its abundance in the normal human intestine[33]. The current results indicated that the therapeutic effect of *L. acidophilus* did not increase in a concentration-dependent manner, but revealed that a moderate-dose concentration (10^6 CFU/10 g) provided the most alleviation of UC symptoms, as evidenced by the significant reductions in DAI and tissue damage scores. Most importantly, this moderate-dose restored UC-related parameters to the levels in non-UC healthy control mice.

Our detailed analyses of the proximal and distal colonic intestinal flora provided further insights into the relationship between the therapeutic effects observed and the concentration of Lactobacillus in the lesion. The symptoms' resolution mediated by the moderate-dose of *L. acidophilus* was accompanied by distinct and significant changes in the distal colon bacterial profile (*i.e.*, increases in Lactobacilli and Bifidobacteria, and decreases in *S. aureus*). Since UC lesions frequently involve the proximal colon and rectum, and the *L. acidophilus* intervention used in this study mainly affected the distal colonic bacterial flora, it is possible that the different therapeutic efficacies observed for the various probiotics in previous studies may result from effects in specific tissues.

In the current study, the number of Lactobacilli detected in specific lesions did not increase in conjunction with increased concentrations of the administered *L. acidophilus*. Since the Lactobacilli population is composed of many species, such as *L. acidophilus*, *L. casei*, *L. plantarum* and *L. bulgaricus*, researchers have started to investigate the therapeutic effects on UC related to the individual species[12,34]. Similarly, efficacy studies on the Lactobacilli compound bacteria VSL#3 have been carried out, and their results confirmed that the combined panel of *Lactobacillus* sp. in this compound is beneficial for treating inflammatory bowel diseases[15,35]. We hypothesize that, in the intestine, interactions between *L. acidophilus* and other local Lactobacilli (at low concentrations) may serve to promote each other mutually. It is possible then that increasing the concentration of *L. acidophilus* (*via* administration of the probiotic supplement) may serve to intensify this mutual promotion. However, the dose of the *L. acidophilus* supplement is important; too high of a dose may instead create an imbalance between the different *Lactobacillus* sp. and disrupt the mutual promotion, thereby leading to a decrease in the total number of the beneficial Lactobacilli. Gaining a detailed understanding of the mechanisms of the interactions between *Lactobacillus* sp. will provide important insights into the related roles in UC pathogenesis.

The optimal dose of *L. acidophilus* will promote the growth of endogenous probiotics, such as Bifidobacteria, and inhibit the growth of pathogenic bacteria, such as *S. aureus*. The moderate-dose of *L. acidophilus* in the current study increased the amount of other probiotics and reduced the amounts of the pathogenic species. However, it appears that simply modifying the dose of *L. acidophilus* will not be sufficient for designing a supplemental regimen with optimal efficacy as we also found that the concentration of endogenous Lactobacilli in the lesion is relatively positively correlated with the efficacy.

In conclusion, administration of *L. acidophilus* supplement at a dose of 10^6 CFU/10 g body weight provides optimal therapeutic effect on experimental colitis in a mouse model. The treatment-induced relief of UC symptoms was correlated with changes in the concentrations of endogenous Lactobacilli and other probiotic and pathogenic bacteria in the distal colon. Future studies should aim to determine the mechanisms underlying the interactions between *L. acidophilus* and other endogenous bacteria, as well as molecular effects on the host immune system, both of which may identify novel manipulable targets to further increase the therapeutic efficacy of this approach.

COMMENTS

Background

Ulcerative colitis (UC) is a nonspecific chronic inflammatory bowel disease with a high rate of recurrence. The etiological factors of UC remain largely unknown, but a large number of studies have demonstrated that the intestinal microorganism environment plays an important role in the development of UC.

Research frontiers

Despite the increased incidence of UC, there remains a distinct lack of efficacious and non-invasive methods of treatment. Besides surgical intervention, which is performed in the later stages of UC, the primary therapies are drug-based, with the most frequently administered agents being salazosulfamide, glucocorticoids and immunosuppressant probiotics. Yet all of these drugs produce significant side-effects that limit patient compliance, which may actually promote the high relapse rates. Because probiotics are beneficial (and predominant) components of the normal intestinal flora, they represent promising therapeutic agents for UC; yet, to date, no study has reported the comparative efficacies of different kinds of probiotics or different doses in UC.

Innovations and breakthroughs

Although probiotics are beneficial to the human body, the supplement preparation of these living bacteria is not static and their dynamic activities, such as proliferation and secreted signaling mechanisms, may influence the therapeutic effects at different sites within the host system. It is necessary to identify the most suitable probiotic type for use a supplemental therapy for UC, as well as the optimal dose that will replenish the endogenous probiotics and inhibit any latent pathogenic species.

Applications

By determining the most suitable concentration of *Lactobacillus acidophilus* (*L. acidophilus*) for use as a supplemental probiotic treatment of UC, and providing novel insights into the relationship between *L. acidophilus* and the other endogenous flora, this study not only promotes the clinical potential for probiotic treatment but also expands the base of knowledge about UC pathogenesis.

Terminology

Inflammatory bowel diseases, such as UC and Crohn's disease, are nonspecific chronic inflammatory conditions with highly complex etiologies. The dextran sodium sulfate-induced mouse model of experimental colitis is similar to human UC.

Peer review

Grammatical errors should be corrected but this manuscript will provide a good addition to the medical literature.

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P- Reviewer Leitman M **S- Editor** Zhai HH **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5357

World J Gastroenterol 2013 August 28; 19(32): 5357-5364
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia

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Supported by Project of the National Key Technologies R and D Program in the 11th Five-Year Plan, No. 2007BAI04B01 and partially supported by Xi'an-Janssen Pharmaceutical Ltd
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Received: March 13, 2013 Revised: June 15, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

AIM: To study the evolution of gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia (FD).

METHODS: From June 2008 to November 2009, a total of 1049 patients with FD (65.3% female, mean age 42.80 ± 11.64 years) who visited the departments of gastroenterology in Wuhan, Beijing, Shanghai, Guangzhou, and Xi'an, China were referred for this study. All of the patients fulfilled the Rome Ⅲ criteria for FD. Baseline demographic data, dyspepsia symptoms, anxiety, depression, sleep disorder, and drug treatment were assessed using self-report questionnaires. Patients completed questionnaires at baseline and after 1, 3, 6 and 12 mo follow-up. Comparison of dyspepsia symptoms between baseline and after follow-up was explored using multivariate analysis of variance of repeated measuring. Multiple linear regression was done to examine factors associated with outcome, both longitudinally and horizontally.

RESULTS: Nine hundred and forty-three patients (89.9% of the original population) completed all four follow-ups. The average duration of follow-up was 12.24 ± 0.59 mo. During 1-year follow-up, the mean dyspeptic symptom score (DSS) in FD patients showed a significant gradually reduced trend ($P < 0.001$), and similar differences were found for all individual symptoms ($P < 0.001$). Multiple linear regression analysis showed that sex ($P < 0.001$), anxiety ($P = 0.018$), sleep disorder at 1-year follow-up ($P = 0.019$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$), and prokinetic use during 1-year follow-up ($P = 0.035$) were horizontally associated with DSS at 1-year follow-up. No relationship was found longitudinally between DSS at 1-year follow-up and patient characteristics at baseline.

CONCLUSION: Female sex, anxiety, and sleep disorder, weight loss, consulting a physician and prokinetic use during 1-year follow-up were associated with outcome of FD.

Key words: Functional dyspepsia; Gastrointestinal symptoms; Dyspeptic symptom score; *Helicobacter py-*

lori infection; Postprandial distress syndrome; Epigastric pain syndrome; Rome Ⅲ criteria

Core tip: This is a prospective study with Chinese patients to explore the clinical course of functional dyspepsia (FD), and evaluate the potential risk factors associated with it, using the Rome Ⅲ criteria both longitudinally and horizontally. The sample size in this study was large and there was a good response rate. The mean dyspepsia symptom score for both total and individual symptoms showed a significant gradually reduced trend. Female sex, anxiety, and sleep disorder, weight loss, consulting a physician and prokinetic use during 1-year follow-up were associated with the outcome of FD.

Yu J, Liu S, Fang XC, Zhang J, Gao J, Xiao YL, Zhu LM, Chen FR, Li ZS, Hu PJ, Ke MY, Hou XH. Gastrointestinal symptoms and associated factors in Chinese patients with functional dyspepsia. *World J Gastroenterol* 2013; 19(32): 5357-5364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5357.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5357

INTRODUCTION

Functional dyspepsia (FD) is a highly prevalent gastrointestinal disorder that is defined by the presence of symptoms thought to originate in the gastroduodenal region, without identifiable cause and diagnosed by routine tests[1,2]. According to the Rome Ⅲ criteria, patients are classified as postprandial distress syndrome (PDS) or epigastric pain syndrome (EPS) based upon the predominant symptom (*i.e.*, postprandial fullness, early satiation, or epigastric pain, and burning)[1]. The reported prevalence of FD symptoms varies between 19% and 41%[3]. FD has a significant impact on quality of life and imposes a substantial economic burden on society due to costs of physician visits, medication, and absenteeism[3,4].

The course of FD is always chronic, with a relapsing-remitting pattern, and has been poorly studied[5]. In spite of the prevalence of FD being stable over time, the reverse in symptom status is high[6]. Many studies have reported that a significant number of patients with FD improve or become asymptomatic over time, suggesting that a proportion of patients go into symptom remission, but the rates of symptom disappearance varies widely[5,7].

The pathophysiology of FD also remains poorly understood and is likely to be multifactorial[8]. Many pathogenic factors have been proposed for FD including genetic, environmental, pathological, and psychological factors[9]. Psychosocial factors such as depression, anxiety and stressful life events (*e.g.*, history of abuse) are considered to play a role in the development of FD[10,11]. A relationship between *Helicobacter pylori* (*H. pylori*) infection and FD has also been reported[12]. Similarly, several studies have demonstrated that sleep disorder is associated with functional gastrointestinal disorders such as irritable bowel syndrome, gastroesophageal reflux disease, and FD[13-15]. Nevertheless, it is not clear whether such pathogenic factors affect the clinical course of FD.

Accordingly, this longitudinal study followed up a group of Chinese patients with FD over 1 year. We aimed to explore the evolution of FD symptoms, and evaluate the potential risk factors both longitudinally and horizontally.

MATERIALS AND METHODS

Patient selection

A total of 1049 patients with FD (364 male and 685 female, aged 20-79 years, mean 42.80 ± 11.64 years) who fulfilled the Rome Ⅲ criteria were enrolled. These were outpatients who visited departments of gastroenterology in five cities in China (Wuhan, Beijing, Shanghai, Guangzhou, and Xi'an) from June 2008 to November 2009.

The patients had one or more dyspeptic symptoms, including troublesome postprandial fullness, early satiation, epigastric pain, or epigastric burning for the past 3 mo, with symptom onset at least 6 mo before diagnosis. All of the FD patients had undergone upper gastrointestinal endoscopy, abdominal ultrasound, and/or barium meal X-ray examination in a tertiary hospital. In all cases, there was no evidence of organic, systemic, or metabolic disease that was likely to explain the symptoms.

Patients were excluded if they: (1) had upper gastrointestinal organic diseases such as esophagitis, peptic ulcer, or peptic neoplasm that were found by gastroscopy or barium meal X-ray examination and abdomen ultrasonography; (2) had chronic diseases such as diabetes mellitus, hyperthyroidism, scleroderma, chronic renal failure, or congestive heart failure; (3) had a history of abdominal surgery; or (4) were pregnant or preparing to conceive a child, or lactating during the study period.

Data collection and synthesis

Baseline data: All 1049 FD patients were asked to finish a self-report questionnaire face to face. To ensure content validity and usability, physicians were trained initially to give instructions to patients and did not intervene with the patients' medical management.

The baseline self-reported questionnaire included several clinical variables involving demographics (age, sex, height, weight, and marital status), tobacco and alcohol use, educational level, economic situation, life satisfaction, physical labor, *H. pylori* status, severity and frequency of each dyspepsia symptom, bowel symptom comorbidity, psychosocial factors (anxiety and depression), sleep disorder, major mental stimulation, history of abuse and drug treatment (prokinetics, gastric mucosa protectants, antacids, anti-*H. pylori* therapy, and traditional Chinese medicine). The data for dyspepsia and bowel symptoms were collected using a Chinese version of the

validated Rome Ⅲ diagnostic questionnaire for adult functional gastrointestinal disease[16]. This questionnaire has been repeatedly tested and carefully validated[17].

Follow-up data: FD patients were asked to visit the department of gastroenterology to finish a follow-up questionnaire at 1, 3, 6 and 12 mo after the first visit. The follow-up questionnaire was the same as the baseline questionnaire, but did not include some details such as sex, educational level, economic situation, life satisfaction, physical labor, major mental stimulation, and history of abuse.

Diagnosis of *H. pylori* infection

At upper gastrointestinal endoscopy, biopsies were acquired and processed for rapid urease test. A $^{13}C/^{14}C$-urea breath test was also used to assess *H. pylori* status.

Definition of body mass index

Body mass index (BMI) was calculated and categorized as weight (kg)/height (m^2) according to World Health Organization recommendations.

Economic situation

Economic situation was classified as rich, sufficient, well-off and poor according to the expending percentage for food in whole income as < 1/5, < 1/3, 1/2, and > 1/2.

Educational level

Educational level was divided into seven: illiteracy, elementary school, junior high school, high school, junior college, university, and graduate and above. If the patients were illiterate or had finished elementary school education, they were judged as having a low level of education. If the patients had completed junior high school or high school education, they were regarded as having a medium level of education. Patients who had completed junior college education or above were considered to have a high level of education.

Tobacco and alcohol use

Current smokers were defined as individuals smoking cigarettes and having no other former tobacco use. Alcohol use was defined as consumption of > 100 g/wk alcohol.

Assessment of dyspeptic symptoms

Dyspeptic symptoms that were recorded and assessed included postprandial fullness, early satiation, epigastric pain, epigastric burning, belching, nausea, vomiting, and bloating. Each symptom was graded and scored on a Likert scale according to its severity as follows: 0, absent; 1, mild (not influencing daily activities); 2, relevant (diverting from but not urging modifications in daily activities); and 3, severe (influencing daily activities markedly enough to urge modifications). Frequency of each symptom was also graded as follows: 1, occurring < 1 d/mo; 2, occurring 1 d/mo; 3, occurring 2-3 d/mo; 4, occurring 1 d/wk; 5, occurring > 1 d/wk; and 6, occurring every day. The score for a single dyspeptic symptom was an aggregate of frequency and severity ratings, ranging from 0 to 9. Dyspeptic symptoms score (DSS) was assessed by summing the score of eight dyspepsia symptoms.

Psychosocial factors (anxiety and depression) and sleep disorder

The questionnaires used for assessment of psychological factors and sleep disorder were established according to a Chinese version of the Validated Rome Ⅲ Psychosocial Alarm Questionnaire for functional gastrointestinal disease[16,18]. In previous studies these questionnaires have been used to assess the psychological factors and sleep status of Chinese patients[19].

For problems related to anxiety and depression in the past 3 mo, the patients answered the question: Did you feel nervous irritable or depressed (yes/no)? If patients chose yes, they had to answer the next question about how often they felt nervous irritable or depressed: 1, occasionally; 2, sometimes; 3, frequently; 4, most of the time. Patients felt nervous irritable or depressed frequently or most of the time, indicating that anxiety or depression was present[16]. In the present study, we judged "nervous irritability" or "depression" occurring frequently or most of the time as "anxiety state" or "depression state".

Subjective sleep disorder in patients was measured with one question (yes/no). Symptoms of sleep disorder included trouble falling asleep, shallow sleep/dreaminess, sleep time < 6 h, early morning awakening, and daytime sleepiness.

History of abuse

A history of abuse in patients was measured with a question as follows: Have you ever been abused (yes/no)? If patients chose yes, they stated whether the abuse was physical or mental.

Statistical analysis

All statistical analyses were assessed using SPSS for Windows version 13. A two-sided P value < 0.05 was regarded as statistically significant. Data are presented as mean ± SD. To assess whether those who completed all four follow-ups were representative of the original study population, we compared the baseline characteristics between the follow-up population and those who were lost to follow-up, using Pearson's χ^2 test (categorical variables), Mann-Whitney U test (ordinal variables, such as BMI) and t test (continuous variables). Comparison between all individual dyspepsia symptoms at initial visit and at the four follow-ups was explored using multivariate analysis of variance (MANOVA) of repeated measuring. Univariate association measures between patient characteristics (baseline as well as 1-year follow-up) and DSS at 1-year follow-up were calculated using Pearson'

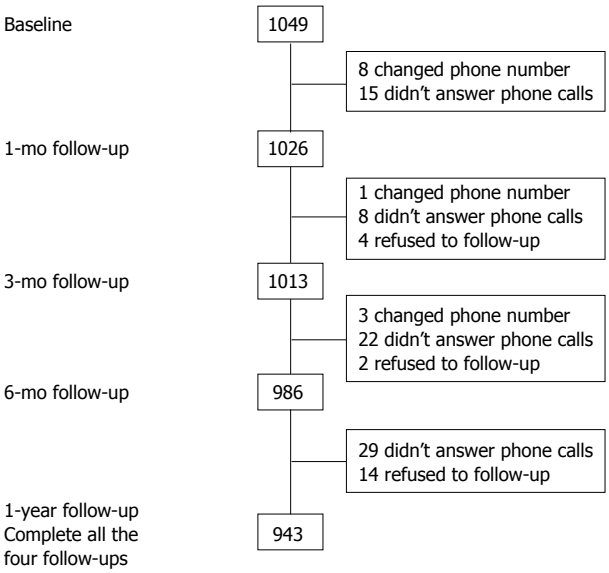

Figure 1 Flowchart of study participants.

s correlation and non-parametric one-way ANOVA. Risk factors associated with DSS at final follow-up, both longitudinally and horizontally, were determined by performing multiple linear regressions.

RESULTS

Patient characteristics and response rate

Of the 1049 FD patients originally enrolled, 1026 patients (97.8% of the baseline sample) completed the 1-mo study; 1013 patients (96.6% of the baseline sample) completed the 3-mo study; 986 patients (94.0% of the baseline sample) completed the 6-mo study; and 943 patients (89.9% of the baseline sample) completed the 1-year study (Figure 1).

The 943 patients who completed the baseline and all four follow-up questionnaires were included in this study. The average duration of follow-up was 12.24 ± 0.59 mo. The mean age of the follow-up population was 42.99 ± 11.74 years, and 603 (63.9%) were female; 176 (18.7%) had bowel symptom comorbidity, and 230 (24.4%) were positive for *H. pylori*. Men, alcohol users, those with higher educational level and better economic situation, and those who had consulted a physician were significantly more likely to be successfully followed up ($P < 0.05$ for all analyses) (Table 1).

Comparison of dyspepsia symptoms between baseline and at follow-up

The mean DSS in FD patients at baseline, and 1, 3 and 6 mo and 1 year follow-up was 22.05 ± 9.89, 14.04 ± 9.38, 12.05 ± 9.09, 10.08 ± 8.89 and 8.97 ± 8.62, respectively. This means that during 1 year follow-up, the mean DSS in FD patients showed a significant gradually reduced trend and all pairwise comparisons were statistically significant (all $P < 0.001$).

Table 1 Demographics and baseline characteristics of patients who completed all four follow-ups compared with those who were lost to follow-up

Characteristic	Complete all four follow-ups (n = 943)	Lost to follow-up (n = 106)	P value
Sex (female)	603 (63.9)	82 (77.4)	0.006
Smoker	202 (21.4)	16 (15.1)	0.128
Alcohol user	278 (29.5)	15 (14.2)	0.001
Marital status (married)	815 (86.4)	95 (89.6)	0.357
Physical labor			0.537
High	34 (3.6)	3 (2.8)	
Medium	238 (25.2)	31 (29.2)	
Low	671 (71.2)	72 (67.9)	
Life satisfaction			
High	171 (18.1)	13 (12.3)	0.292
Medium	732 (77.6)	90 (84.9)	
Low	40 (4.2)	3 (2.8)	
Educational level			0.011
High	336 (35.6)	28 (26.4)	
Medium	455 (48.3)	51 (48.1)	
Low	152 (16.1)	27 (25.5)	
Economic situation			0.03
Rich	40 (4.2)	3 (2.8)	
Sufficient	405 (42.9)	34 (32.1)	
Well-off	469 (49.7)	67 (63.2)	
Poor	29 (3.1)	2 (1.9)	
BMI (kg/m^2)			0.071
Obesity	138 (14.6)	10 (9.4)	
Normal	709 (75.2)	81 (76.4)	
Thin	96 (10.2)	15 (14.2)	
Bowel symptom	176 (18.7)	23 (21.7)	0.45
H. pylori status (positive)	230 (24.4)	22 (20.8)	0.14
Consulting a physician	908 (96.3)	91 (85.8)	< 0.001
Mean age (yr)	42.99 ± 11.74	41.11 ± 10.65	0.116
DSS	22.05 ± 9.89	20.25 ± 10.77	0.079

Data are expressed as absolute numbers (percentage) or mean ± SD. Significant variables in italic/bold ($P < 0.05$); BMI: Body mass index calculated; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

Similar differences were observed for all individual symptoms (Figure 2). The mean symptom scores for both postprandial fullness and belching during 1 year follow-up showed a significant reduced trend and all pairwise comparisons were statistically significant (all $P < 0.001$).

The mean symptom scores for early satiation, nausea and bloating during 1 year follow-up decreased significantly and all pairwise comparisons were statistically different (all $P < 0.001$, except for the difference between 3 and 6 mo follow-up, $P = 0.037$, $P = 0.035$, $P = 0.102$, and the difference between 6 mo and 1 year follow-up, $P = 0.333$, $P = 0.034$, $P = 0.213$).

There was a marked decreased trend in mean symptom scores for both epigastric pain and epigastric burning during 1-year follow-up, and there were significant differences in all pairwise comparisons (all $P < 0.001$, except for the difference between 6 mo and 1 year follow-up, $P = 0.401$, $P = 0.028$).

The mean symptom scores for vomiting was reduced markedly, with all pairwise comparisons showing a significant difference (all $P < 0.001$, except for the difference

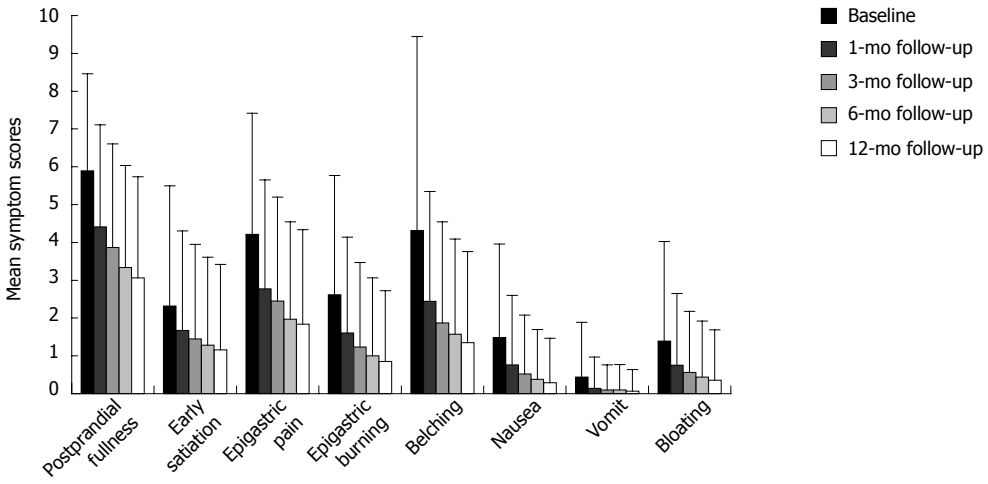

Figure 2 Comparison of dyspeptic symptoms between initial visit and at four follow-ups of repeated measures.

between 1 mo and 3 mo follow-up, $P = 0.330$; the difference between 3 mo and 6 mo follow-up, $P = 0.959$; the difference between 6 mo and 1 year follow-up, $P = 1.0$).

Factors associated with DSS at final follow-up (1-year follow-up)

Longitudinal associations: For patient characteristics at baseline, univariate correlates analysis revealed that history of abuse was associated with DSS at 1-year follow-up ($P = 0.025$), while no association was found for other variables such as sex, age, BMI, anxiety, depression, sleep disorder, *H. pylori* status, DSS at baseline, and drug treatment before baseline (Table 2). Multiple linear regression analysis showed no relationship between DSS at 1-year follow-up and patient characteristics at baseline (Table 2).

Horizontal associations: For patient characteristics at 1-year follow-up, univariate correlates analysis found that age ($P < 0.001$), alcohol consumption ($P = 0.024$), anxiety ($P < 0.001$), depression ($P < 0.001$), sleep disorder ($P < 0.001$), bowel symptoms ($P < 0.001$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$), prokinetic use ($P < 0.001$), gastric mucosa protectant use ($P < 0.001$), antacid use ($P < 0.001$), and traditional Chinese medicine use ($P < 0.001$) were significantly associated with DSS (Table 3).

Multiple linear regression analysis showed that sex ($P < 0.001$), anxiety ($P = 0.018$), sleep disorder ($P = 0.019$), weight loss ($P < 0.001$), consulting a physician ($P < 0.001$) and prokinetic use ($P = 0.035$) were significantly associated with DSS, while age, depression, alcohol consumption, bowel symptoms, and use of gastric mucosa protectants, antacids and traditional Chinese medicine were not associated with it (Table 3).

DISCUSSION

To the best of our knowledge, this is the first published prospective study with Chinese patients to explore the clinical course of FD, and evaluate potential risk factors associated with FD, using the Rome Ⅲ criteria, both longitudinally and horizontally. We selected a large group of FD patients from five cities in China. After their initial visit, patients were followed up at 1, 3 and 6 mo and 1 year.

The sample size was large and we had a good response rate to all parts of the study. We compared the baseline characteristics between the follow-up population and those who were lost to follow-up. We found that men, alcohol users, those with higher educational level and better economic situation, and those who had consulted a physician were significantly more likely to be successfully followed up. This is consistent with previous reports[20,21]. There were some demographic differences between responders and non-responders, but the magnitude of these differences was small and the individuals included in our follow-up study were broadly representative of the original enrolled FD patients, suggesting that the results of our study are persuasive.

The novel finding of our study was that the total DSS in FD patients showed a significant gradually reduced trend during 1 year follow-up, and similar differences were found for all individual symptoms. It seems that patients feel much better at the final follow-up and complain of less discomfort. Several previous studies reported improved symptoms during a period of follow-up, which is in line with our findings[5]. Kindt *et al*[22], in a 5-year follow-up study, found that about half of FD patients reported disappeared or improved symptoms. Pajala *et al*[23] observed a marked reduction in DSS in FD patients in Finland after 1 year follow-up. Heikkinen *et al*[24], in a long-term perspective study, also concluded that the stability of the symptom-based subgroups over time was poor. However, all of these studies only compared two time points, while our study compared five points.

Furthermore, we identified risk factors that influenced the clinical course of FD. Over the past decade, the correlation between psychological factors and func-

Table 2 Longitudinal associations between functional dyspepsia patients' baseline characteristics and dyspeptic symptom score at 1-year follow-up

Variable (at baseline)	Longitudinal associations							
	Univariate correlates			Multiple linear regression				
	r[1]	F[2]	P	B	β	t	P	R^2_{Model}
Sex[3]		3.489	0.062	0.161	0.009	0.208	0.835	0.019
Age	0.002		0.959	-0.007	-0.009	-0.224	0.823	
BMI	0.007		0.835	-0.024	-0.009	-0.248	0.804	
Smoking		1.894	0.169	1.228	0.058	1.368	0.172	
Alcohol consumption		0.02	0.887	-0.665	-0.035	-0.876	0.381	
Major mental stimulation		1.288	0.257	-0.421	-0.013	-0.365	0.715	
History of abuse		5.022	0.025[4]	-3.774	-0.062	-1.762	0.078	
Anxiety		0.106	0.745	0.897	0.030	0.777	0.437	
Depression		0.937	0.333	-1.549	-0.044	-1.157	0.248	
Sleep disorder		1.831	0.176	-0.683	-0.040	-1.131	0.259	
Bowel symptom		0.17	0.680	0.442	0.020	0.580	0.562	
H. pylori status		0.461	0.631	-0.269	-0.027	-0.782	0.434	
DSS	-0.014		0.667	-0.015	-0.018	-0.502	0.616	
Treatments in the previous months before baseline								
Consulting a physician		0.753	0.386	0.468	0.027	0.804	0.421	
Prokinetic use		0.077	0.782	-0.598	-0.034	-0.933	0.351	
Gastric mucosa protectant use		1.407	0.236	0.611	0.034	0.971	0.332	
Antacid use		1.381	0.24	0.589	0.034	0.906	0.365	
Anti-*H. pylori* therapy		1.014	0.314	0.431	0.018	0.539	0.590	
Traditional Chinese medicine use		0.005	0.945	-0.025	-0.001	-0.042	0.966	

[1]Pearson's correlation; [2]Non-parametric one-way ANOVA; [3]Male = 0, female = 1 reference category: female; [4]Significant variables ($P < 0.05$). BMI: Body mass index; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

Table 3 Horizontal associations between functional dyspepsia patients' baseline characteristics and dyspeptic symptom score at 1-year follow-up

Variable (at 1-yr follow-up)	Horizontal associations							
	Univariate correlates			Multiple linear regression				
	r[1]	F[2]	P	B	β	t	P	R^2_{Model}
Sex[3]		3.489	0.062	0.585	0.033	6.356	< 0.001[4]	0.98
Age	0.139		< 0.001[4]	-0.002	-0.002	-0.456	0.649	
Time of follow-up (mo)	-0.035		0.288	-0.121	-0.008	-1.837	0.067	
Smoking		1.109	0.293	-0.242	-0.009	-1.533	0.126	
Alcohol consumption		5.132	0.024[4]	0.204	0.008	1.346	0.179	
Anxiety		13.257	< 0.001[4]	0.292	0.015	2.373	0.018[4]	
Depression		27.452	< 0.001[4]	-0.052	-0.002	-0.392	0.695	
Sleep disorder		69.219	< 0.001[4]	0.216	0.012	2.346	0.019[4]	
Bowel symptom		51.053	< 0.001[4]	0.185	0.007	1.377	0.169	
H. pylori status		1.563	0.210	-0.035	-0.003	-0.759	0.448	
Treatments during 1-yr follow-up period								
Consulting a physician		224.718	< 0.001[4]	0.893	0.051	9.168	< 0.001[4]	
Prokinetic use		59.340	< 0.001[4]	0.200	0.012	2.113	0.035[4]	
Gastric mucosa protectant use		22.857	< 0.001[4]	-0.014	-0.001	-0.147	0.883	
Antacid use		19.313	< 0.001[4]	-0.023	-0.001	-0.266	0.790	
Anti-*H. pylori* therapy		1.825	0.177	-0.075	-0.003	-0.635	0.526	
Traditional Chinese medicine use		50.799	< 0.001[4]	-0.062	-0.004	-0.755	0.450	
Weight loss during 1-yr follow-up period	0.988		< 0.001[4]	1.040	0.961	186.775	< 0.001[4]	

[1]Pearson's correlation; [2]Non-parametric one-way ANOVA; [3]Male = 0, female = 1 reference category: female; [4]Significant variables ($P < 0.05$). BMI: Body mass index; *H.pylori*: *Helicobacter pylori*; DSS: Dyspeptic symptom score.

tional gastrointestinal disorders has been confirmed in several clinical case-control studies[25,26]. Koloski *et al*[27] found that anxiety was an evident independent predictor for FD. Aro *et al*[28], in a Swedish population-based study, showed that anxiety but not depression was linked to FD and PDS but not to EPS. In the present study, anxiety at 1-year follow-up was also found to be horizontally associated with DSS, which is in keeping with previous studies.

Sleep disorder is a common phenomenon in all FD patients. Dyspeptic symptoms can interfere with sleep, and disrupted sleep may also potentially exacerbate FD

symptoms due to the hyperalgesic effect of sleep loss. Cremonini *et al*[14], in a study involving 3228 respondents, found that sleep disturbances were linked to both upper and lower gastrointestinal symptoms in the general population. Lacy *et al*[15] revealed that there was a relationship between FD and sleep disorder, and sleep disorder in FD patients appeared to be associated with symptom severity and higher levels of anxiety. We also discovered an association between sleep disorder at 1-year follow-up and FD outcome.

Recent cross-sectional population studies discovered that weight loss correlated most strongly with early satiety, followed by nausea and vomiting[29]. A longitudinal study in Belgium found that weight loss was independently associated with FD-specific quality of life at follow-up, and there was a trend association between weight loss and DSS at follow-up[22]. In this study, we did not have information about weight difference between dyspepsia symptom onset and initial visit. However, we collected data on patients' weight at baseline and at final follow-up, and observed that weight loss during 1 year follow-up was independently associated with DSS.

We showed an association between sex and FD outcome, indicating that women may have higher DSS at 1-year follow-up than men have, which is consistent with a cross-sectional study in Taiwan[30]. There was no association between *H. pylori* status and DSS at 1-year follow-up, which is similar to another prospective 2-year follow-up study from Taiwan[31].

An important finding in our study was that many individuals reported persistent symptoms despite consultation and prokinetic use during 1 year follow-up. Similarly, two recent studies have also reported persistence of symptoms in drug-treated patients[32,33]. It is probable that patients consulting a physician have the most severe symptoms, and they often take prescribed drugs on an on-demand basis. In addition, most individuals (n = 511, 54% of follow-up patients) in our study had a prescription of prokinetic during 1 year follow-up. These may be the reasons why patients consulting a physician and taking prokinetic still have continuous symptoms or even more severe symptoms.

In bivariate analysis, we also found a correlation between history of abuse and DSS at 1-year follow-up, which was similar to several other characteristics (*i.e.*, age, alcohol consumption, depression, bowel symptoms, and use of gastric mucosa protectants, antacids and traditional Chinese medicine during 1 year follow-up period). However, this correlation was not found in multiple linear regression analysis, indicating that it was weak.

In conclusion, in this large sample of individuals with FD, 89.9% of patients completed all four follow-ups, and the average duration of follow-up was 12.24 ± 0.59 mo. During 1 year follow-up, the total DSS in FD patients showed a significant gradually reduced trend, and similar differences were found for all individual symptoms. Female sex, anxiety, sleep disorder, weight loss, consulting a physician, and prokinetic use during 1 year follow-up were associated with outcome. Our study described the fluctuations in symptoms and found that several associated factors affected outcome. We believe that these findings provide evidence for the role of psychosocial factors in determining long-term clinical course in patients with FD. In the future, more research is needed to confirm and extend our study.

COMMENTS

Background

Functional dyspepsia (FD) is a chronic functional gastrointestinal disorder, and it has a significant impact on quality of life and imposes a substantial economic burden on society. However, the clinical course and risk factors for FD remain poorly studied.

Research frontiers

In the present study, the authors selected a large group of FD patients from five cities in China and explored the clinical course and risk factors for FD both longitudinally and horizontally.

Innovations and breakthroughs

Some cross-sectional population studies have discovered several risk factors associated with FD. This is believed to be the first follow-up study showing that the total dyspeptic symptoms score and single symptom score in FD patients present a significant gradually reduced trend, and female sex, anxiety, sleep disorder, weight loss, consulting a physician, and prokinetic use during 1-year follow-up were associated with outcome.

Applications

The study described the fluctuations in dyspeptic symptoms and found several factors were associated with the outcome. This may provide evidence for the role of psychosocial factors in determining the long-term clinical course of patients with FD.

Terminology

FD is a highly prevalent gastrointestinal disorder that is defined by the presence of symptoms thought to originate in the gastroduodenal region, without identifiable cause by routine diagnostic methods.

Peer review

This is a follow-up study evaluating the clinical course and potential risk factors for FD. This is an interesting article discussing an important area in functional gastrointestinal disorders.

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P- Reviewers Devanarayana NM, de la Roca-Chiapas JM
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5365

World J Gastroenterol 2013 August 28; 19(32): 5365-5376
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Systematic review and meta-analysis of laparoscopy-assisted and open total gastrectomy for gastric cancer

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Supported by The key project grant from the Science and Technology Department of Zhejiang Province, China, No. 2011C3036-2

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Received: March 29, 2013 Revised: May 23, 2013
Accepted: July 9, 2013
Published online: August 28, 2013

Abstract

AIM: To evaluate the safety and efficacy of laparoscopy-assisted total gastrectomy (LATG) and open total gastrectomy (OTG) for gastric cancer.

METHODS: A comprehensive search of PubMed, Cochrane Library, Web of Science and BIOSIS Previews was performed to identify studies that compared LATG and OTG. The following factors were checked: operating time, blood loss, harvested lymph nodes, flatus time, hospital stay, mortality and morbidity. Data synthesis and statistical analysis were carried out using RevMan 5.1 software.

RESULTS: Nine studies with 1221 participants were included (436 LATG and 785 OTG). Compared to OTG, LATG involved a longer operating time [weighted mean difference (WMD) = 57.68 min, 95%CI: 30.48-84.88; $P < 0.001$]; less blood loss [standard mean difference (SMD) = -1.71; 95%CI: -2.48 - -0.49; $P < 0.001$]; earlier time to flatus (WMD= -0.76 d; 95%CI: -1.22 - -0.30; $P < 0.001$); shorter hospital stay (WMD = -2.67 d; 95%CI: -3.96 - -1.38, $P < 0.001$); and a decrease in medical complications (RR = 0.41, 95%CI: 0.19-0.90, $P = 0.03$). The number of harvested lymph nodes, mortality, surgical complications, cancer recurrence rate and long-term survival rate of patients undergoing LATG were similar to those in patients undergoing OTG.

CONCLUSION: Despite a longer operation, LATG can be performed safely in experienced surgical centers with a shorter hospital stay and fewer complications than open surgery.

Key words: Laparoscopy; Total gastrectomy; Gastric cancer; Complications; Meta-analysis

Core tip: This study evaluated the safety and efficacy of laparoscopy-assisted total gastrectomy (LATG) and open total gastrectomy (OTG) for gastric cancer through systematic review and meta-analysis. The existing research shows that LATG is safe and feasible, which can achieve similar lymph node dissection effects as OTG, characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG.

Chen K, Xu XW, Zhang RC, Pan Y, Wu D, Mou YP. Systematic review and meta-analysis of laparoscopy-assisted and open total gastrectomy for gastric cancer. *World J Gastroenterol* 2013; 19(32): 5365-5376 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5365.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5365

INTRODUCTION

Since it was first reported in 1994[1], laparoscopy-assisted distal gastrectomy (LADG) for gastric cancer has undergone rapid development and gained popularity in the past 20 years. Compared to traditional open gastrectomy, most studies have reported that LADG can achieve better cosmesis, shorter hospital stay, faster postoperative recovery, and better postoperative quality of life[2-6]. However, laparoscopy-assisted total gastrectomy (LATG) is technically demanding and the incidence of upper gastric carcinoma is relatively low in East Asia[7,8]. Therefore, although LADG has been accepted worldwide for tumors located in the lower stomach, LATG for upper and middle gastric cancer has not been generalized. In fact, there are only a few reports on the technical feasibility and safety of LATG and its long-term oncologic outcomes[9-12]. Although several meta-analyses and systematic reviews have been published for LADG[13-19], such studies have not been conducted for the potential benefits and disadvantages of LATG.

In order to assess accurately the current status of LATG, we strictly limited inclusion criteria by focusing exclusively on LATG and carried out a comprehensive meta-analysis. We believe that such research will contribute to a more systematic and objective evaluation of the safety of the LATG in cancer treatment.

MATERIALS AND METHODS

Search strategy

We searched PubMed, Cochrane Library, Web of Science and BIOSIS Previews for literature comparing LATG and open total gastrectomy (OTG) published between January 1995 and March 2013, and broadened the search range by browsing the related summary, methods, and references of retrieved articles. The following keywords were used: "laparoscopy", "laparoscopic", "gastric cancer", "gastric carcinoma", and "gastrectomy". The language of the publications was confined to English. Two investigators reviewed the titles and abstracts, and assessed the full text to establish eligibility.

Inclusion and exclusion criteria

All clinical studies should meet the following criteria for the meta-analysis: (1) published in English with data comparing LATG and OTG; (2) clear case selection criteria, containing at least the following information: the number of cases, surgical methods and perioperative data; and (3) if there was overlap between authors or centers, the higher quality or more recent literature were selected. However, articles from the same authors or centers but with different patient cohorts were included. The papers containing any of the following were excluded: (1) totally laparoscopic, laparoscopic hand-assisted, or robot-assisted gastrectomy; (2) non-gastric carcinoma cases; (3) palliative resection cases; and (4) extent of lymphadenectomy was not required for grouping in this study, but the articles with significant differences between the two groups in the extent of lymphadenectomy were excluded.

Data extraction and quality assessment

Two authors independently extracted the data using a unified datasheet, and decided upon the controversial issues through discussion. Extracted data included: author, study period, geographical region, number of patients, operating time, blood loss, number of retrieved lymph nodes, proximal and distal margin distance, time to flatus, time to oral intake, length of hospital stay, morbidity and mortality. Postoperative complications were classified as medical (cardiovascular, respiratory, or metabolic events; nonsurgical infections; deep venous thrombosis; and pulmonary embolism) or surgical (any anastomotic leakage or fistula, any complication that required reoperation, intra-abdominal collections, wound complications, bleeding events, pancreatitis, ileus, delayed gastric emptying, and anastomotic stricture). This classification system is based on the Memorial Sloan-Kettering Cancer Center complication reporting system[20]. If necessary, the first authors were contacted to retrieve further information. Selected documents were rated according to the grading of the Centre of Evidence-Based Medicine (CEBM, Oxford, United Kingdom; http://www.cebm.net), which, in brief, assigns level 1 to randomised controlled trials (RCTs), level 2 to cohort studies, level 3 to case-control studies, level 4 to case series or poor quality observational study and level 5 to expert opinion.

Statistical analysis

The meta-analysis was performed in line with recommendations from the Cochrane Collaboration and the Quality of Reporting of Meta-Analyses guidelines[21,22]. Continuous variables, when both means and standard deviations were presented, were assessed using weighted mean difference (WMD) or standard mean difference (SMD), the postoperative morbidity and mortality were analyzed using the risk ratio (RR), and the risk difference (RD) was used to evaluate cancer recurrence because there may be no recurrence events in either groups during follow-up. When heterogeneity test showed no significant differences ($P > 0.05$), we used a fixed-effects model to calculate the summary statistics. When the heterogeneity test showed statistically significant differences ($P < 0.05$), we used a random effects model based on the DerSimonian and Laird method. Subgroup analysis of intraoperative outcomes, such as operating time, blood loss, and number of retrieved lymph nodes, was conducted for the number of LATG cases performed (40 cases were used as a cut-point), because the learning curve may have an impact on the operative outcomes. Potential publication bias was determined by conducting informal visual inspection of funnel plots based on the complications. Data analyses were performed using Review Manage Version 5.1 (RevMan 5.1) software downloaded from Cochrane Library. $P < 0.05$ was considered statistically significant.

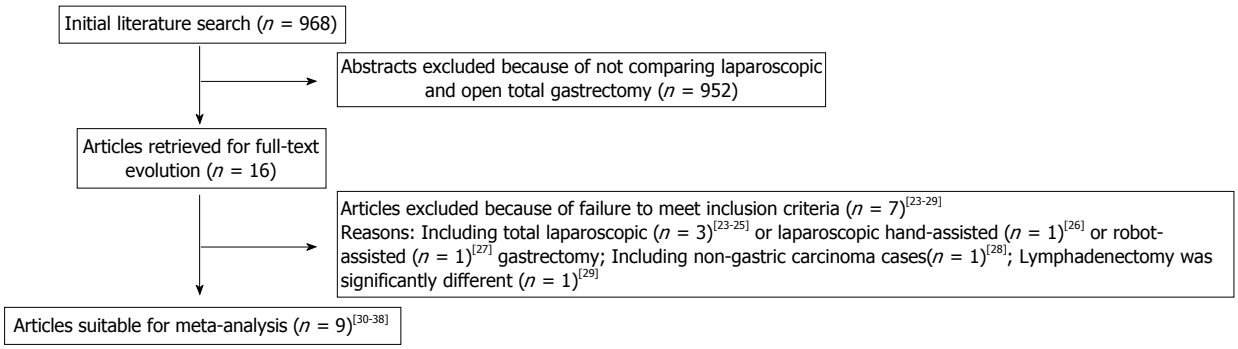


Figure 1 Flow chart of literature search strategies.

Table 1 Characteristics of included studies

Ref.	Nation	Study type	Study period	Sample size		Stage	Level of lymphadenectomy	Follow-up (mo)		Level of evidence
				LATG	OTG			LATG	OTG	
Kim *et al*[30]	South Korea	Retro	2004-2006	27	33	EC + AC	D1 + α/β, D2	NR	NR	2b
Mochiki *et al*[31]	Japan	Retro	1999-2007	20	18	EC + AC	D1 + β	31 (3-60)	46 (13-60)	2b
Sakuramoto *et al*[32]	Japan	Retro	2003-2007	30	44	EC + AC	D1 + β, D2	30		4
Kawamura *et al*[33]	Japan	Retro	2003-2008	46	35	EC	D2	NR	NR	4
Du *et al*[34]	China	Retro	2005-2009	82	94	AC	D2	25 (2-44)		2b
Kim *et al*[35]	South Korea	Pros	2009-2010	63	127	EC + AC	D2	NR	NR	2b
Kunisaki *et al*[36]	Japan	Pros	2002-2008	27	30	EC + AC	D1 + β	NR	NR	3b
Eom *et al*[37]	Korea	Retro	2003-2008	100	348	EC + AC	D2	52.6 (0.3-95.7)		4
Guan *et al*[38]	China	Pros	2007-2010	41	56	EC + AC	D2	NR	NR	3b

Retro: Retrospective observational study; Pros: Prospective observational study; EC: Early gastric cancer; AC: Advanced gastric cancer; NR: Not reported; LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy.

RESULTS

Studies selected

The initial search strategy retrieved 968 publications in English. After the titles and abstracts were reviewed, papers without comparison of LATG and OTG were excluded, which left 16 comparative studies, seven[23-29] of which did not meet the inclusion criteria and were excluded. This left a total of nine comparative observational studies[30-38]. A flow chart of the search strategies is illustrated in Figure 1.

Study characteristics and quality

A total of 1221 patients were included in the analysis with 436 undergoing LATG (35.7%) and 785 undergoing OTG (64.3%). Only one study reported a case converted to open surgery because of extensive abdominal adhesions[38]. Regarding the tumor stage, only one study was limited to early stage cancer[33]. In another study, only patients with advanced gastric cancer were described[34]. The other seven studies included both populations. All studies had Asian data from Japan, South Korea and China. In the included studies, four studies was considered as level of evidence 2b, two studies as level of evidence 3b, and the remaining three as level of evidence 4 (according to the grading of the CEBM). The characteristics and methodological quality assessment scores of the included studies are shown in Table 1.

Intraoperative effects

Most of the studies considered suitable for the meta-analysis reported a longer operating time for LATG than for OTG. The mean operating time of LATG was 57.68 min longer than for OTG (WMD = 57.68 min; 95%CI: 30.48-84.88, $P < 0.001$) (Figure 2A). Two studies[31,32] used grams but the others[33-36,38] used milliliters as the unit of measurement for intraoperative blood loss, therefore, SMD was used to synthesize the data. The intraoperative blood loss was lower in LATG than OTG (SMD = -1.71; 95%CI: -2.48 - -0.94, $P < 0.001$) (Figure 2B). All studies contained the number of retrieved lymph nodes. The difference in the mean number of retrieved lymph nodes between LATG and OTG was not significant in the pooled data (WMD = -1.41; 95%CI: -3.15 - 0.32, $P = 0.11$) (Figure 2C). Two studies described the proximal and distal margin distances[35,38]. Meta-analysis of the distal margin distance showed no significant difference between the two groups (WMD = 0.46 cm; 95%CI: -0.40 - 1.32, $P = 0.29$). However, the proximal margin distance of OTG was longer than that of LATG with a marginal difference (WMD = -0.40 cm; 95%CI: -0.82 - 0.02, $P = 0.06$). All intraoperative effect outcomes are summarized in Table 2.

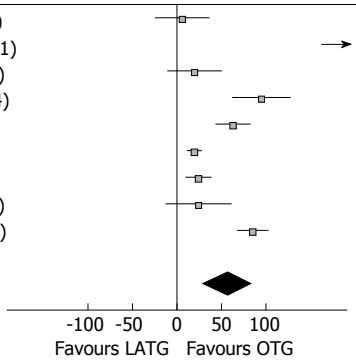
Subgroup analysis for learning curve

The overall effects of operating time and blood loss remained unchanged in subgroups, although performing

A

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Mochiki *et al*[31]	254	44.7	20	248	50.9	18	10.8%	6.00 (-24.61, 36.61)
Kim *et al*[30]	527.5	95.7	27	320.9	75.8	33	9.4%	206.60 (162.19, 251.01)
Kawamura *et al*[33]	291.9	59.4	46	272.1	76.8	35	10.8%	19.80 (-10.89, 50.49)
Sakuramoto *et al*[32]	313	81	30	218	53	44	10.6%	95.00 (62.06, 127.94)
Du *et al*[34]	275	78	82	212	51	94	11.8%	63.00 (43.22, 82.78)
Kim *et al*[35]	150.8	31.2	63	131.2	21.6	127	12.4%	19.60 (11.03, 28.17)
Guan *et al*[38]	235.7	38.5	41	211.5	33.2	56	12.1%	24.20 (9.55, 38.85)
Kunisaki *et al*[36]	286.4	68	27	262.1	74.9	30	10.2%	24.30 (-12.80, 61.40)
Eom *et al*[37]	283.7	84.1	100	198.5	59.7	348	11.9%	85.20 (67.56, 102.84)
Total (95%CI)			436			785	100.0%	57.68 (30.48, 84.88)

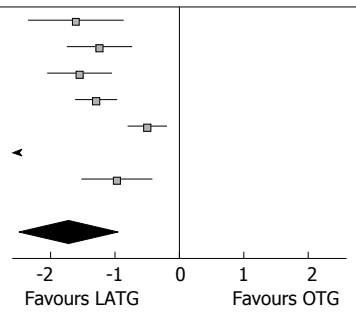
Heterogeneity: Tau² = 1535.02; χ^2 = 127.63, *df* = 8 (*P* < 0.00001); I^2 = 94%
Test for overall effect: *Z* = 4.16 (*P* < 0.0001)



B

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Mochiki *et al*[31]	299	223.6	20	758	330.9	18	13.5%	-1.61 (-2.35, -0.87)
Sakuramoto *et al*[32]	134	98	30	407	270	44	14.5%	-1.24 (-1.75, -0.73)
Kawamura *et al*[33]	54.9	45.3	46	304.3	237.3	35	14.5%	-1.55 (-2.05, -1.05)
Du *et al*[34]	156	112	82	339	162	94	15.1%	-1.29 (-1.62, -0.97)
Kim *et al*[35]	179.7	123.8	63	272.7	209.6	127	15.1%	-0.50 (-0.80, -0.19)
Guan *et al*[38]	104.2	42.9	41	355.6	51.3	56	13.0%	-5.20 (-6.05, -4.35)
Kunisaki *et al*[36]	155	138.8	27	422.4	350.4	30	14.3%	-0.97 (-1.52, -0.42)
Total (95%CI)			309			404	100.0%	-1.71 (-2.48, -0.94)

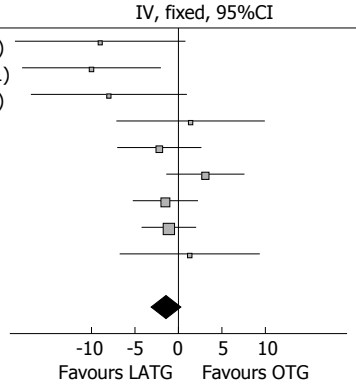
Heterogeneity: Tau² = 0.99; χ^2 = 109.49, *df* = 6 (*P* < 0.00001); I^2 = 95%
Test for overall effect: *Z* = 4.36 (*P* < 0.0001)



C

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, fixed, 95%CI
Mochiki *et al*[31]	26	13.4	20	35	17	18	3.1%	-9.00 (-18.81, 0.81)
Kim *et al*[30]	27.2	15.7	27	37.2	15.7	33	4.7%	-10.00 (-17.99, -2.01)
Sakuramoto *et al*[32]	43.2	17.2	30	51.2	22.1	44	3.7%	-8.00 (-16.97, 0.97)
Kawamura *et al*[33]	48.5	16.3	46	47.1	21.5	35	4.1%	1.40 (-7.14, 9.94)
Du *et al*[34]	34.2	13.5	82	36.4	19.1	94	12.8%	-2.20 (-7.04, 2.64)
Kim *et al*[35]	38.7	15.7	63	35.6	13.1	127	14.9%	3.10 (-1.40, 7.60)
Eom *et al*[37]	48.3	16.4	100	49.8	18.4	348	21.4%	-1.50 (-5.25, 2.25)
Guan *et al*[38]	23.1	8	41	24.2	7.5	56	30.5%	-1.10 (-4.24, 2.04)
Kunisaki *et al*[36]	38.1	13.9	27	36.8	17.1	30	4.6%	1.30 (-6.76, 9.36)
Total (95%CI)			436			785	100.0%	-1.41 (-3.15, 0.32)

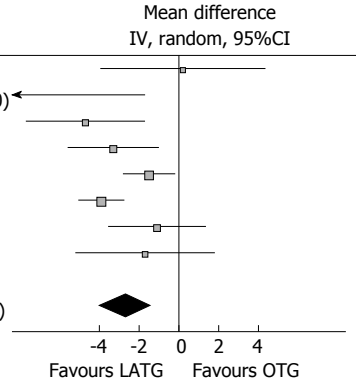
Heterogeneity: χ^2 = 13.67, *df* = 8 (*P* = 0.09); I^2 = 41%
Test for overall effect: *Z* = 1.60 (*P* = 0.11)



D

Study or subgroup	LATG Mean	LATG SD	LATG Total	OTG Mean	OTG SD	OTG Total	Weight	Mean difference IV, random, 95%CI
Kim *et al*[30]	16.2	7.1	27	16	9.3	33	7.1%	0.20 (-3.95, 4.35)
Mochiki *et al*[31]	19	13.4	20	29	12.7	18	2.2%	-10.00 (-18.30, -1.70)
Sakuramoto *et al*[32]	13.5	2.7	30	18.2	9.6	44	10.9%	-4.70 (-7.70, -1.70)
Kawamura *et al*[33]	15.5	3.3	46	18.8	6.3	35	14.5%	-3.30 (-5.59, -1.01)
Kim *et al*[35]	8.1	3.8	63	9.6	5.3	127	20.9%	-1.50 (-2.82, -0.18)
Guan *et al*[38]	9.7	2.2	41	13.6	3.6	56	21.9%	-3.90 (-5.06, -2.74)
Kunisaki *et al*[36]	14.5	3.5	27	15.6	5.8	30	13.5%	-1.10 (-3.56, 1.36)
Eom *et al*[37]	12.6	15.5	100	14.3	16.7	348	9.0%	-1.70 (-5.21, 1.81)
Total (95%CI)			354			691	100.0%	-2.67 (-3.96, -1.38)

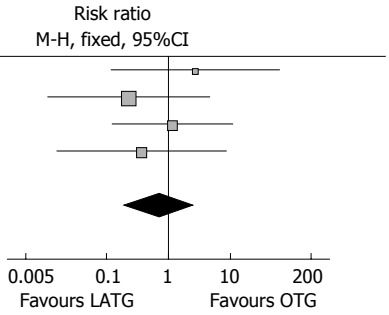
Heterogeneity: Tau² = 1.63; χ^2 = 16.04, *df* = 7 (*P* = 0.02); I^2 = 56%
Test for overall effect: *Z* = 4.05 (*P* < 0.0001)



E

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	1	20	0	18	9.3%	2.71 (0.12, 62.70)
Du *et al*[34]	0	82	2	94	41.5%	0.23 (0.01, 4.70)
Eom *et al*[37]	1	100	3	348	23.8%	1.16 (0.12, 11.03)
Kunisaki *et al*[36]	0	27	1	30	25.3%	0.37 (0.02, 8.69)
Total (95%CI)		229		490	100.0%	0.72 (0.20, 2.57)
Total events	2		6			

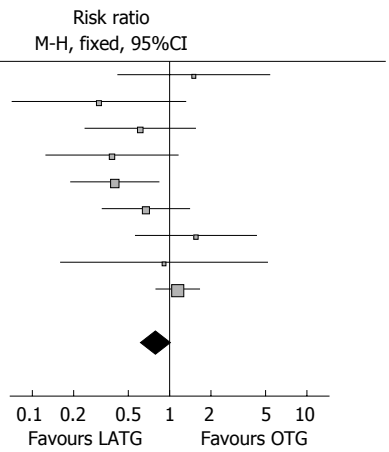
Heterogeneity: χ^2 = 1.58, *df* = 3 (*P* = 0.66); I^2 = 0%
Test for overall effect: *Z* = 0.51 (*P* = 0.61)



F

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	5	20	3	18	2.9%	1.50 (0.42, 5.41)
Kim *et al*[30]	2	27	8	33	6.5%	0.31 (0.07, 1.32)
Sakuramoto *et al*[32]	5	30	12	44	8.8%	0.61 (0.24, 1.56)
Kawamura *et al*[33]	4	46	8	35	8.2%	0.38 (0.12, 1.16)
Du *et al*[34]	8	82	23	94	19.4%	0.40 (0.19, 0.84)
Kim *et al*[35]	8	63	24	127	14.4%	0.67 (0.32, 1.41)
Kunisaki *et al*[36]	7	27	5	30	4.3%	1.56 (0.56, 4.33)
Guan *et al*[38]	2	41	3	56	2.3%	0.91 (0.16, 5.20)
Eom *et al*[37]	27	100	82	348	33.2%	1.15 (0.79, 1.67)
Total (95%CI)		436		785	100.0%	0.79 (0.61, 1.02)
Total events	68		168			

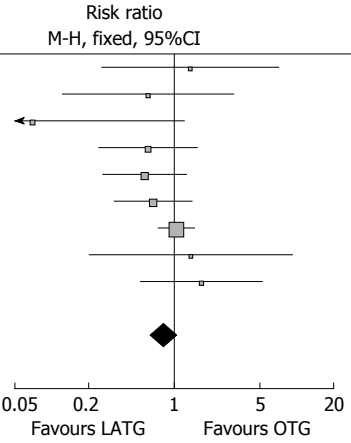
Heterogeneity: χ^2 = 13.42, *df* = 8 (*P* = 0.10); I^2 = 40%
Test for overall effect: *Z* = 1.83 (*P* = 0.07)



G

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	3	20	2	18	2.1%	1.35 (0.25, 7.19)
Kim *et al*[30]	2	27	4	33	3.6%	0.61 (0.12, 3.09)
Kawamura *et al*[33]	0	46	5	35	6.2%	0.07 (0.00, 1.22)
Sakuramoto *et al*[32]	5	30	12	44	9.6%	0.61 (0.24, 1.56)
Du *et al*[34]	8	82	16	94	14.7%	0.57 (0.26, 1.27)
Kim *et al*[35]	8	63	24	127	15.7%	0.67 (0.32, 1.41)
Eom *et al*[37]	29	100	97	348	42.8%	1.04 (0.73, 1.48)
Guan *et al*[38]	2	41	2	56	1.7%	1.37 (0.20, 9.30)
Kunisaki *et al*[36]	6	27	4	30	3.7%	1.67 (0.53, 5.28)
Total (95%CI)		436		785	100.0%	0.83 (0.64, 1.08)
Total events	63		166			

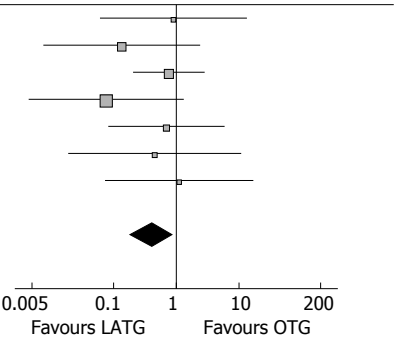
Heterogeneity: χ^2 = 8.14, *df* = 8 (*P* = 0.42); I^2 = 2%
Test for overall effect: *Z* = 1.39 (*P* = 0.16)



H

Study or subgroup	LATG Events	LATG Total	OTG Events	OTG Total	Weight	Risk ratio M-H, fixed, 95%CI
Mochiki *et al*[31]	1	20	1	18	5.0%	0.90 (0.06, 13.36)
Kim *et al*[30]	0	27	4	33	19.3%	0.13 (0.01, 2.40)
Kawamura *et al*[33]	4	46	4	35	21.5%	0.76 (0.20, 2.83)
Du *et al*[34]	0	82	7	94	33.1%	0.08 (0.00, 1.32)
Eom *et al*[37]	1	100	5	348	10.6%	0.70 (0.08, 5.89)
Guan *et al*[38]	0	41	1	56	6.0%	0.45 (0.02, 10.83)
Kunisaki *et al*[36]	1	27	1	30	4.5%	1.11 (0.07, 16.91)
Total (95%CI)		343		614	100.0%	0.41 (0.19, 0.90)
Total events	7		23			

Heterogeneity: χ^2 = 3.84, *df* = 6 (*P* = 0.70); I^2 = 0%
Test for overall effect: *Z* = 2.21 (*P* = 0.03)



I

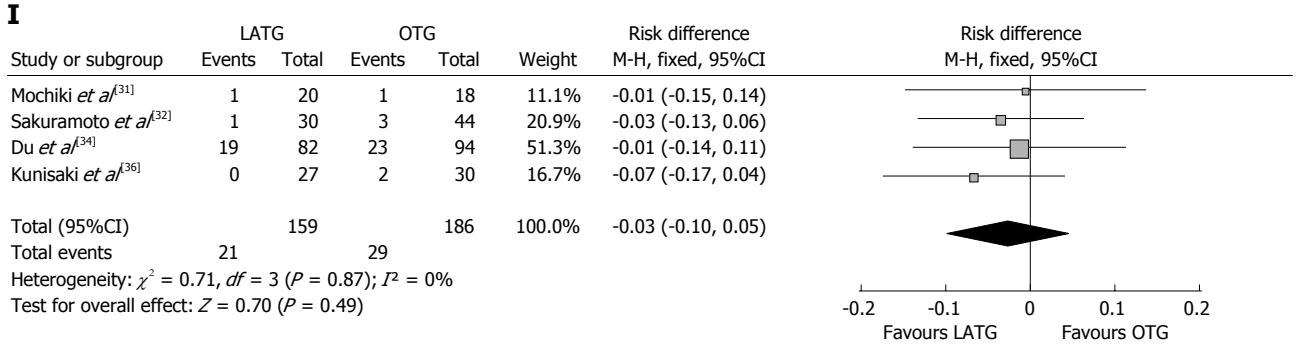


Figure 2 Meta-analysis. A: The pooled data: operating time; B: The pooled data: intraoperative blood loss; C The pooled data: number of retrieved lymph nodes; D: The pooled data: duration of hospital stay; E: The pooled data: mortality; F: The pooled data: overall postoperative complications; G: The pooled data: surgical complications; H: The pooled data: medical complications; I: The pooled data: recurrences.

Table 2 Results of meta-analysis

Outcome	No. of study	Sample size		Heterogeneity (P, I^2)	Overall effect size	95%CI of overall effect	P value
		LATG	OTG				
Operating time (min)	9	436	785	< 0.001, 94%	WMD = 57.68	30.48-84.88	< 0.001
Blood loss	7	309	404	< 0.001, 95%	SMD = -1.71	-2.48 - -0.94	< 0.001
Retrieved lymph nodes	9	436	785	0.09, 41%	WMD = -1.41	-3.15 - 0.32	0.11
Proximal margin (cm)	2	163	475	1.00, 0%	WMD = -0.40	-0.82 - 0.02	0.06
Distal margin (cm)	2	163	475	0.67, 0%	WMD = 0.46	-0.40 - 1.32	0.29
Analgesics given	4	221	300	< 0.001, 93%	SMD = -0.86	-1.62 - -0.11	0.02
Duration of fever (d)	2	112	138	0.47, 0%	WMD = -1.58	-1.80 - -1.37	< 0.001
Time to first flatus (d)	7	316	419	< 0.001, 91%	WMD = -0.76	-1.22 - -0.30	0.001
Time to oral intake (d)	4	161	257	0.04, 63%	WMD = -0.81	-1.26 - -0.35	< 0.001
Hospital stay (d)	8	354	691	0.02, 56%	WMD = -2.67	-3.96 - -1.38	< 0.001
Overall complications	9	436	785	0.10, 40%	RR = 0.79	0.61-1.02	0.07
Surgical complications	9	436	785	0.42, 2%	RR = 0.83	0.64-1.08	0.16
Medical complications	7	343	614	0.70, 0%	RR = 0.41	0.19-0.90	0.03
Mortality	4	229	490	0.66, 0%	RR = 0.72	0.20-2.57	0.61

WMD: Weighted mean difference; SMD: Standard mean difference; LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy.

> 40 LATG cases demonstrated a moderate reduction in operating time and blood loss. Lymph node retrieval was lower in the subgroup with < 40 LATG cases performed (WMD = -6.12; 95%CI: -10.42 - -1.81, P = 0.005). However, there was no difference when > 40 LATG procedures were performed (WMD = -0.50; 95%CI: -2.4 - 1.39, P = 0.60). The outcomes of subgroup analysis are summarized in Table 3.

Postoperative outcome

Flatus is one of the outcome measures for evaluating postoperative recovery of gastrointestinal functions. The mean time to first flatus was shorter in LATG than in OTG (WMD= -0.76 d; 95%CI: -1.22 - -0.30, P = 0.001), as was the time to restart oral intake after surgery (WMD = -0.81 d; 95%CI: -1.26 - -0.35, P < 0.001). Postoperative analgesic consumption was less in LATG than in OTG (SMD = -0.86; 95%CI: -1.62 - -0.11, P = 0.02). A shorter hospital stay was also observed in the LATG group (WMD = -2.67 d; 95%CI: -3.96 - -1.38, P < 0.001) (Figure 2D). All postoperative outcomes are summarized in Table 2.

Two studies reported inflammatory response index such as white blood cell (WBC) count and C-reactive protein (CRP)[32,33]. A significantly lower WBC count for LATG compared with OTG was found on postoperative days 1, 3, 7[32,33] and 10[33], and lower CRP for LATG was found on postoperative day 1 in both studies[32,33].

Mortality was described in four studies, and there was no significant difference in postoperative mortality (RR = 0.72, 95%CI: 0.20-2.57, P = 0.61) (Figure 2E). Morbidity was addressed and specified in all studies with exception of Kunisaki's study[36]. We contacted the authors of this study to get information about the specific complications. The rate of overall postoperative complications was lower for LATG with a marginal difference (RR = 0.79, 95%CI: 0.61-1.02, P = 0.07) (Figure 2F). Visual inspection of the funnel plot revealed symmetry, indicating no serious publication bias (Figure 3). After further analysis, surgical complications were similar between the two groups (RR = 0.83, 95%CI: 0.64-1.08, P = 0.16) (Figure 2G), without the exception of any specific complications such as anastomotic leakage, intra-abdominal collections, bleeding, or anastomotic stricture. LATG was associated, however, with a significant reduction in medical complications (RR = 0.41, 95%CI: 0.19-0.90, P = 0.03) (Figure 2H) with a

Table 3 Subgroup analysis for learning curve using a cut-point of 40 laparoscopy-assisted total gastrectomy cases

Outcome	No. of study	Sample size		Heterogeneity (P, I^2)	Overall effect size	95%CI of overall effect	P value
		LATG	OTG				
Operating time (min)							
< 40 LATG cases	4	104	125	< 0.001, 95%	WMD = 81.99	1.47-162.5	0.05
> 40 LATG cases	5	332	660	< 0.001, 93%	WMD = 42.53	16.23-68.82	0.002
Blood loss							
< 40 LATG cases	3	77	92	0.40, 0%	SMD = -1.22	-1.55 - -0.88	< 0.001
> 40 LATG cases	4	232	312	< 0.001, 97%	SMD = -2.07	-3.35 - -0.79	0.002
Retrieved lymph nodes							
< 40 LATG cases	4	104	125	0.20, 36%	WMD = -6.12	-10.42 - -1.81	0.005
> 40 LATG cases	5	332	660	0.47, 0%	WMD = -0.50	-2.4 - 1.39	0.60

LATG: Laparoscopy-assisted total gastrectomy; OTG: Open total gastrectomy; WMD: Weighted mean difference; SMD: Standard mean difference.

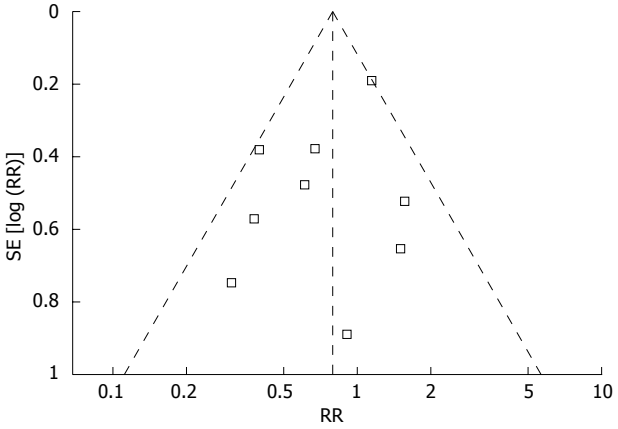


Figure 3 Funnel plot of the overall postoperative complications.

possible contribution from respiratory complications (RR = 0.34, 95%CI: 0.11-1.03, P = 0.06). The outcomes of mortality and morbidity are summarized in Table 2.

Recurrence and long-term survival rate

During the follow-up period, cancer recurrence was observed in four studies[31,32,34,36]. The recurrence risk in LATG was 13.2% (21/159) and 15.6% (29/186) in OTG, but the difference between LATG and OTG was not significant (RD = -0.03, 95%CI: -0.10-0.05, P = 0.49) (Figure 2I).

Three trials reported the long-term survival rate[31,36,37]. Mochiki *et al*[31] have reported that there was no significant difference in the cumulative or disease-specific 5-year survival rates between LATG and OTG (cumulative: 95% in LATG, 90.9% in OTG; disease-specific: 100% in LATG, 91.7% in OTG, P = 0.81). Eom *et al*[37] have reported that the survival rates were similar between groups; the hazard ratio of LATG *vs* OTG was 0.43 (95%CI: 0.15-1.20; P = 0.107) for overall survival and 0.47 (95%CI: 0.19-1.18; P = 0.106) for disease-free survival. Kunisaki *et al*[36] also have reported that there was no significant differences in overall and disease-specific survival between groups.

DISCUSSION

RCTs are the most ideal tools for meta-analysis. However, no RCTs on LATG have yet been conducted because the history and popularity of LATG are insufficient compared with LADG, due to the fact that it is difficult to dissect splenic hilar lymph nodes and mobilize the esophagus under a laparoscope, while it is demanding to perform Roux-en-Y esophagojejunostomy through mini-laparotomy. Thus, our meta-analysis synthesized the existing observational studies with strictly limiting inclusion and exclusion criteria. The included studies were primarily derived from the countries with the most widespread use of laparoscopic gastrectomy (four from Japan, three from Korea, and two from China), and all published in the past 5 years (2008-2012), and the total number of cases incorporated in the study was 1221. The meta-analysis conducted based on this point will contribute a more comprehensive and objective evaluation for the current LATG surgical status.

Similar to most reports comparing laparoscopic and open surgery in many different clinical situations, the intraoperative blooding in the LATG group was less than that in the OTG group, as is the need for transfusions. The reduced length of incision wound and the application of energy-dividing devices, such as the Harmonic Scalpel and Ligasure, contribute to the reduction in blood loss. Lack of blood is a common problem faced by many hospitals, especially in developing countries such as China. Therefore, less-invasive laparoscopic surgery can reduce the clinical requirement for blood and lower the rate of complications associated with blood transfusions such as virus infection and allergic reaction. In addition, some researchers have suggested that transfusions are associated with increased perioperative mortality and morbidity[39].

Regarding the operating time, LATG is more time-consuming than OTG. LATG combined with lymphadenectomy is a complex operation and needs a lot of technical expertise. Almost all of the studies included in this meta-analysis demonstrated prolonged operating time in LATG, despite significant heterogeneity. Learning curve which related to the surgeon's experience, familiarity with instruments, and assistant compliance could influence some outcomes studied, such as operating time or lymph node retrieval[40]. Because several of the researches included in this study reported on their initial experience, so

we performed a subgroup analysis using 40 LATG cases as a cut-point and demonstrated a moderate reduction in LATG operating time. Another reason for the prolonged operating time for LATG may be related to the reconstructive step, which is more difficult to complete through minilaparotomy than open surgery because of the narrow operating window for manual suture or anvil insertion and application of other instruments, especially in obese patients. To overcome these potential problems, various modified techniques have been reported, such as laparoscopic purse-string suture technique using Endo Stitch (Covidien, Mansfield, MA, United States)[41], Endo-PSI (Hope Electronics Co., Ltd, Shenzhen, China)[42], or a hemi-double stapling technique[43]. Another two intracorporeal reconstruction methods may be most representative; one using a transorally inserted anvil (OrVil; Covidien) to make an end-to-side esophagojejunostomy[44], the other using linear staplers to make a side-to-side anastomosis[45]. These methods not only avoid auxiliary incision, but also help to simplify the procedure of reconstruction and shorten the operating time[46,47].

The inflammatory stress reaction is an inevitable outcome of operative trauma and is an important index for measuring its extent. Some studies have compared inflammatory cytokines such as interleukin (IL)-6, IL-10 and CRP in plasma of patients who have undergone laparoscopic or laparotomic resection for gastroenteric cancer. The postoperative level of IL-6, IL-10 and CRP increased but the levels in the laparoscopic group are significantly lower than in the laparotomic group[48-50]. A meta-analysis of laparoscopic colectomy has also demonstrated that the postoperative IL-6 level of laparotomic group patients was significantly lower than that of laparotomic group[51]. The studies included in this research show that the WBC count and CRP of patients in the LATG group were lower than those in the OTG group, and serum protein was higher[32,33], indicating that LATG imposes few inflammatory stimuli on patients and consumes less protein. Kawamura *et al*[33] have also found that postoperative blood glucose in OTG patients is significantly higher than that in LATG patients when the same amount of calories was ingested, indicating that LATG has a lower effect on sugar metabolism.

The most striking finding was a reduced number of complications in the LATG *vs* OTG group, which may have resulted from a reduction in medical complications. It was conceivable that surgical complications were similar between groups because LATG results in the same organ and lymphatic resection as OTG. However, it is worth noting that some studies have found that there is a high risk of anastomotic stricture after LATG[10,52], whereas our study found morbidity associated with anastomotic stricture was similar between the two groups. Prevention of anastomotic stricture has long been one of the main tasks in total gastrectomy and also should not be ignored in LATG. Some researchers hold that side-to-side esophagojejunostomy could be used to reduce the risk of anastomotic stricture because a larger anastomotic stoma can be made from it[45,53]. Besides, the significantly decreased medical complications could be explained by the reduced invasiveness of the laparoscopic technique and less postoperative pain. We also found that respiratory complications occurred in LATG less often than in OTG, although the difference was not significant (P = 0.06). The pain caused by large incision as well as the use of tension sutures and abdominal bandages after laparotomy can make it difficult for patients to cough, expectorate and perform exercise breathing effectively, thus leading to such complications as pulmonary infection[54]. Pain after surgery was less serious in LATG than in OTG due to the shorter duration or the lower dosage of analgesic application[32-35]. The time to first flatus was also earlier in LATG than in OTG, which indicated a rapid recovery of gastrointestinal function after LATG. Reduced use of analgesic drugs, shortened time of abdominal cavity exposure, alleviated inflammatory reactions, and earlier postoperative activities are considered to be the main reasons for earlier gastrointestinal recovery from LATG; all of which may also contribute to shortening the duration of postoperative hospital stay.

The adequacy of the radical resection should be evaluated by the extent of lymph node dissection performed and the number of harvested lymph nodes, as well as the length of the resection margins. We found that fewer lymph nodes were obtained after LATG than in OTG, even though the difference was not significant. However, the subgroup analysis with 40 cases in LATG showed that the difference was shrinking. The number of laparoscopic lymph nodes dissected was closely related to the level of surgical technique. In recent years, with increasingly mature techniques, some researchers have reported not only a similar number of overall retrieved lymph nodes between LADG and open distal gastrectomy, but also a similar number of specific lymph nodes, such as group 7, 8a, 9, 11p, 12a and 14v, which used to be considered difficult for laparoscopic dissection[55,56]. Splenic hilar lymph node dissection is one of the difficulties in radical total gastrectomy, which is because the splenic vessels run circuitously, and the branches vary substantially and they are in a narrow space at a very deep location. It is easy to cause hemorrhage because of splenic vascular injury or cause spleen ischemia and further necrosis by accidental cutting of the splenic artery branches of when dissecting the lymph nodes in this area. Compared to laparotomy, laparoscopy allows the operator to complete the spleen hilum lymph node dissection under a clear field of view and helps to improve surgical safety[57].

With regard to the length of the resection margin, we found that the proximal margin in LATG was shorter than that of OTG. Such result may relate to the nature for LATG which should resect specimen and make reconstruction all through mini-laparotomy; and it is difficult to pull the proximal stomach using a narrow incision, which may influence the distance of proximal margin. Therefore, patients with smaller neoplasms are more likely to receive LATG instead of OTG, thus allowing

the surgeon to choose a smaller excision extension.

Cancer recurrence and long-term survival rate are two critical outcomes for evaluating surgical interventions in oncological therapy. LATG is not superior to LADG in both history and popularity, and only three studies have compared the long-term survival rate between the two groups[31,36,37], and another two have performed a descriptive analysis of cancer recurrence[32,34]. Based on these data, postoperative cancer recurrence and long-term survival rate in LATG were similar to those in OTG. However, as the cases in the studies included in our analysis were mostly concerned with early gastric cancer, the effect of LATG for early gastric cancer should be affirmed. Some RCTs and meta-analyses have demonstrated that long-term follow-up outcome of laparoscopic gastrectomy for advanced gastric cancer is similar to that of laparotomy[58,59]. Recently, Park *et al*[60] have analyzed the follow-up results of 239 cases of advanced gastric cancer treated with laparoscopic radical gastrectomy. Among these cases, 130 were T_2 stage, 63 were T_3, and 46 were T_4, and the 5-year survival rates were 86.6%, 77.4% and 58.7%, respectively. The result is similar to that for concurrent laparotomy and is encouraging. However, there should be an attitude of caution for laparoscopic resection of advanced gastric cancer because relevant studies and clinical evidence are still deficient.

During our research, a similar article by Haverkamp *et al*[61] was published, which had several limitations. The clinical heterogeneity could have been caused by the different underlying conditions and interventions. It is well known that gastric submucosal tumors (SMTs) such as lymphoma, leiomyosarcoma, and gastrointestinal stromal tumors are significantly different from adenocarcinoma in terms of biological characteristics, clinical diagnosis, and treatment. In our study, only patients who underwent gastrectomy for gastric adenocarcinoma were included, but Haverkamp *et al* included 8 patients undergoing total gastrectomy for *SMTs*; this may influence the reliability the results[28]. The difference in surgical methods is a major cause of clinical heterogeneity. In laparoscopy-assisted gastrectomy (LAG), an incision is almost always required for extracting a relatively large specimen and involves some complicated steps. However, totally laparoscopic gastrectomy (TLG) is considered to be incisionless, except for the trocar wounds, and it is a laparoscopic approach for intracorporeal anastomosis without auxiliary incision and touching the tumor. Hence, these are two different operative methods. Furthermore, some studies have shown that TLG may be less invasive than LAG, with the disadvantage of prolonged operating time[47,62-66]. Therefore, it is inappropriate to pool trials that differ in terms of these two methods in a meta-analysis. However, the existing meta-analysis included a study in which the TLG was performed using a totally laparoscopic method[23]. In addition, for the trials without the mean and standard deviation, Haverkamp *et al* used the median and range to estimate them based on the Hozo method[67]. However, this method may lead to deviation, especially when the sample size is small or the samples exhibit serious skewness. In the study of Topal for example[23], the median intraoperative blood in the laparoscopic group (n = 38) was 10 (5-400) mL, so the estimated mean blood loss was 10 mL. In fact, however, even the minimum mean blood loss could be 15.4 mL, which differed from the estimated value. Besides, since the study by Haverkamp *et al*[61] was published, several clinical observational studies have become available. The larger the number of patients in a meta-analysis, the greater its power to detect a possible treatment effect. Therefore, our comprehensive meta-analysis will contribute to a more systematic and objective evaluation for the safety and cancer treatment of LATG.

In conclusion, the existing research shows that LATG is safe and feasible, which can achieve similar lymph node dissection effects as OTG, characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG. However, most of the published studies were retrospective, the sample sizes were relatively small, most of the cases were early gastric cancer, the follow-up periods were not long enough, and the results exhibited substantial heterogeneity. Therefore, the results mentioned above should be subject to verification by strictly designed, large-sample, multicenter, RCTs.

COMMENTS

Background

Since it was first reported in 1994, laparoscopy-assisted distal gastrectomy (LADG) for gastric cancer has undergone rapid development and gained popularity in the past 20 years. Compared with traditional open gastrectomy, LADG can achieve better cosmesis, shorter hospital stay, faster postoperative recovery, and better postoperative quality of life. Although LADG has been accepted worldwide for tumors located in the lower stomach, laparoscopy-assisted total gastrectomy (LATG) for upper and middle gastric cancer has not been generalized. Although several meta-analyses and systematic reviews have been published for LADG, such studies have not been conducted for the potential benefits and disadvantages of LATG.

Research frontiers

In order to assess accurately the current status of LATG, the authors strictly limited inclusion criteria by focusing exclusively on LATG and carried out a comprehensive meta-analysis. This will contribute to a more systematic and objective evaluation of the safety of the LATG in cancer treatment.

Innovations and breakthroughs

LATG is safe and feasible, which can achieve similar lymph node dissection effects as open total gastrectomy (OTG), characterized by such advantages as less pain, fewer postoperative complications, and rapid recovery, and which is expected to achieve the same effect in oncological treatment as OTG.

Applications

Despite a longer operation, LATG can be performed safely in experienced surgical centers with a shorter hospital stay and fewer complications than open surgery.

Peer review

This is a well written paper which will add a great deal to the literature on the subject. One of the most significant conclusions from this work is the lack of randomised controlled trials surrounding the field. Future research should compare LADG and LATG to further verify the safety and feasibility of LATG.

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P- Reviewers Braga S, Clark J, Zhang WH **S- Editor** Wen LL
L- Editor A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5377

World J Gastroenterol 2013 August 28; 19(32): 5377-5380
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Noninfectious interstitial lung disease during infliximab therapy: Case report and literature review

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Received: March 8, 2013 Revised: May 27, 2013
Accepted: June 1, 2013
Published online: August 28, 2013

Abstract

Pulmonary abnormalities are not frequently encountered in patients with inflammatory bowel diseases. However, lung toxicity can be induced by conventional medications used to maintain remission, and similar evidence is also emerging for biologics. We present the case of a young woman affected by colonic Crohn's disease who was treated with oral mesalamine and became steroid-dependent and refractory to azathioprine and adalimumab. She was referred to our clinic with a severe relapse and was treated with infliximab, an anti-tumor necrosis factor α (TNF-α) antibody, to induce remission. After an initial benefit, with decreases in bowel movements, rectal bleeding and C-reactive protein levels, she experienced shortness of breath after the 5th infusion. Noninfectious interstitial lung disease was diagnosed. Both mesalamine and infliximab were discontinued, and steroids were introduced with slow but progressive improvement of symptoms, radiology and functional tests. This represents a rare case of interstitial lung disease associated with infliximab therapy and the effect of drug withdrawal on these lung alterations. Given the increasing use of anti-TNF-α therapies and the increasing reports of pulmonary abnormalities in patients with inflammatory bowel diseases, this case underlines the importance of a careful evaluation of respiratory symptoms in patients undergoing infliximab therapy.

Key words: Interstitial lung disease; Crohn's disease; Infliximab; Mesalamine; Drug-induced toxicity

Core tip: Safety during anti-tumor necrosis factor (TNF)-α therapy is a major concern. Paradoxical inflammatory and autoimmune phenomena can be induced by this treatment and should always be considered. Interstitial lung disease is an emerging complication often observed early after the beginning of treatment, particularly when combination immunosuppressive regimens are employed. This case demonstrates that interstitial lung disease can also occur later during anti-TNF-α treatment and during monotherapy. Thus, great vigilance is recommended when patients start complaining of any respiratory symptom.

Caccaro R, Savarino E, D'Incà R, Sturniolo GC. Noninfectious interstitial lung disease during infliximab therapy: Case report and literature review. *World J Gastroenterol* 2013; 19(32): 5377-5380 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5377.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5377

INTRODUCTION

The occurrence of pulmonary involvement in patients with inflammatory bowel disease (IBD) was first described in 1976 and has been explained either as a potential extra-intestinal manifestation of the disease itself or as a secondary effect of medications employed to control

inflammation[1-4]. The common embryological origin of both the gastrointestinal tract and the respiratory system could be responsible for the shared antigenicity leading to the pulmonary manifestations. However, noninfectious drug-induced lung disease has been described using sulfasalazine, mesalamine, methotrexate and azathioprine[2,4]. Anti-tumor necrosis factor (TNF)-α agents have also been implicated as a cause of drug-induced interstitial lung disease and account for most of the cases reported in the rheumatology literature[5,6].

We report the case of a noninfectious interstitial pneumonia that occurred during infliximab (IFX) treatment in a young woman with colonic Crohn's disease (CD).

CASE REPORT

A 25-year-old female was diagnosed with left-sided ulcerative colitis (UC) in 2004 (16-year-old) and treated with oral and rectal mesalamine. She required several courses of oral prednisone during the subsequent 4-year follow up. Azathioprine was introduced in 2008 because of steroid dependency; however, despite the optimization of the dosage up to 2.5 mg/kg, the patient never experienced a full clinical remission. Colonoscopy demonstrated a segmental distribution of the ulcerative lesions, and histology confirmed CD. According to these findings, in December 2010, the patient discontinued azathioprine and was screened for biologics. Adalimumab (ADA) was started with an induction regimen followed by maintenance. After 4 mo, the patient was referred for a new disease flare and did not respond to concomitant therapy with 25 mg of prednisone. Biochemical parameters demonstrated thrombocytosis (810 × 10^3/μL) and elevated C-reactive protein (25 mg/L) and fecal lactoferrin (538 μg/mL). The new endoscopic assessment showed moderate activity in the left colon and mild lesions in the cecum and terminal ileum (Simple Endoscopic Score for CD 13). The interval between ADA administrations was then reduced to every week for one month, without any significant clinical or biochemical improvement. ADA was stopped, and IFX was started (5 mg/kg) with concomitant steroid tapering. She improved clinically, and her C-reactive protein levels normalized. After the 5th infusion, the patient reported the onset of shortness of breath and fatigue, without concomitant cough or fever. The patient had no history of asthma, atopy or allergy to medications. Chest X-ray did not demonstrate any significant lesion, and thorax auscultation was normal. In accordance with the lung specialist who preliminarily suspected pulmonary sarcoidosis, the 6th dose of IFX was administered, and the patient was admitted to the Pneumology Unit for monitoring. High-resolution computed tomography (HRCT) of the thorax revealed bilateral shadowing nodules and adjacent interstitial thickening with a predominant distribution in the middle and basal regions and relative sparing of the apices (Figure 1). Pulmonary function tests were compatible with a moderately restrictive pattern, without any oximetric deficiency. Bronchoscopy did not demonstrate any endobronchial abnormality, and a bronchoalveolar lavage fluid analysis was negative for *Pneumocystis carinii*, fungi and alcohol-acid resistant bacilli. Cyto-immunological analysis revealed increased cellularity (213 × 10^6/L) with a decreased percentage of macrophages (66%) and a well-represented component of eosinophils (20%) (Table 1). Transbronchial biopsy showed a mild chronic, nonspecific, non-granulomatous infiltrate and thickening of the basal membrane. A QuantiFERON-TB Gold test, auto-antibodies, serum angiotensin-converting enzyme, serum precipitins and blood cultures were unremarkable. Mesalamine and infliximab were discontinued, and prednisone was started at a dose of 50 mg/d for 7 d and subsequently tapered to 25 mg/d in association with inhalations of budesonide and a long-acting beta2-agonist. Clinical improvement occurred over the following 6 wk, with mild symptoms still present at 8 wk. HRCT performed after 10 wk showed minimal peripheral irregularities in both apices. All the symptoms had subsided by week 14. The spirometric values during the follow up are reported in Table 2.

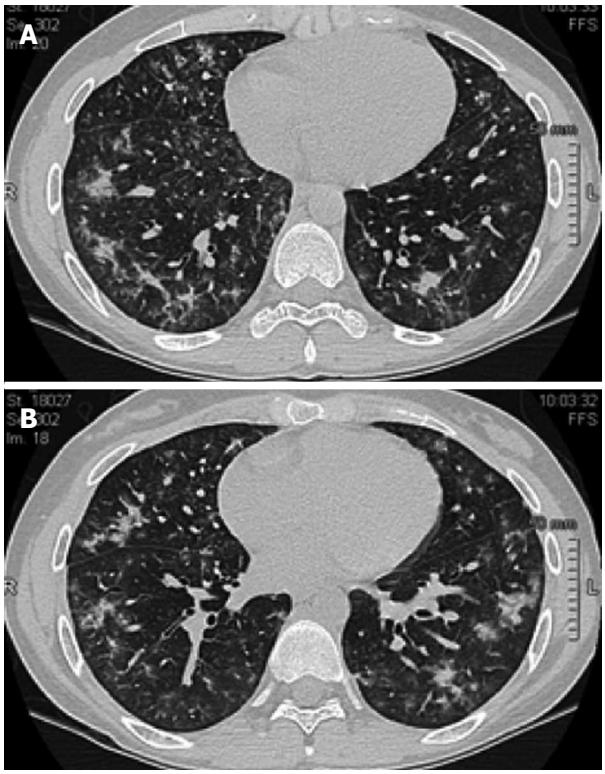

Figure 1 High-resolution computed tomography at hospital admission in the Pneumology Unit. High-resolution computed tomography of the thorax revealed bilateral shadowing nodules and adjacent interstitial (A) thickening with a predominant distribution in the middle and basal regions and relative sparing of the apices (B). R: Right; L: Left.

Table 1 Analysis of the bronchoalveolar lavage fluid

	Cells (10^6/L)	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Patient	213	66%	13%	1%	20%
Ref. values	120-190	85%-93%[1]	5%-12%[1]	2%[1]	< 1%[1]

[1]According to Meyer[16].

Table 2 Spirometric values during the follow up

	4-wk follow up	8-wk follow up	12-wk follow up
FEV1, L	2.03 (58%)	2.24 (64%)	2.56 (73%)
FVC, L	2.08 (52%)	2.33 (58%)	2.62 (66%)
TLC, L	3.04 (56%)	3.63 (67%)	3.73 (69%)
RV, L	0.91 (62%)	1.30 (88%)	0.97 (66%)
FRC, L	1.62 (57%)	2.47 (87%)	2.44 (86%)
DLCO, mL/mmHg per minute	NA	17.3 (58%)	19.0 (64%)

FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity; TLC: Total lung capacity; RV: Residual volume; FRC: Functional residual capacity; DLCO: Diffusion capacity of carbon monoxide; NA: Not available.

DISCUSSION

Although IBDs are pathologic conditions of the gastrointestinal tract, they should be considered as systemic diseases because almost all organs can be involved, although the most frequent extra-intestinal manifestations are articular, dermatologic, ophthalmologic and hepatobiliary[7,8].

Pulmonary involvement can manifest with different patterns[9]. A significant proportion of IBD patients show abnormal functional tests compared to healthy matched controls, suggesting the potential presence of subclinical pulmonary dysfunctions[2,10-13]. In addition and more importantly, HRCT scans performed in 2 series of consecutive IBD patients were pathological in 53.00% and 64.10% of patients, respectively[14,15]. Interestingly, these findings were unrelated to the presence of respiratory symptoms.

Our patient had never previously experienced respiratory symptoms; she did not smoke and did not suffer from asthma or atopy. The previous existence of subclinical respiratory defects cannot be excluded because the patient did not perform a functional respiratory test before the onset of pulmonary symptoms. Nonetheless, it is reasonable to predict the absence of abnormalities because the baseline chest-X ray performed before starting anti-TNF-α therapy was unremarkable.

In addition to IBD-associated pulmonary manifestations, the occurrence of drug-induced effects has to be considered, particularly according to the cyto-immunological analysis of bronchoalveolar lavage fluid, which in our case, was consistent with subacute respiratory illness compatible with either nonspecific interstitial pneumonia or cryptogenic pneumonia[16-18]. Several cases of pulmonary toxicity induced by sulfasalazine and mesalamine have been reported, particularly eosinophilic pneumonia, which is characterized by eosinophilic infiltration of the lungs with or without peripheral eosinophilia[2]. In our patient, we detected normal levels of peripheral eosinophils, and transbronchial biopsy did not reveal an abnormal number of these cells; however, the examination of specimens obtained by fiberoptic bronchoscopy is suboptimal for the diagnosis of interstitial lung diseases. Most of the reported reactions occurred between 2 and 6 mo after the introduction of the drug, with rare cases occurring later on (44 mo)[2,19,20]. Peripheral eosinophilia was often present, and the resolution of symptoms (dyspnea, fever, chest pain, cough) with the discontinuation of the drug was prompt[2]. When our patient first developed shortness of breath, she had been under mesalamine treatment for 8 years. The possibility of mesalamine-induced pneumonia seems unlikely. However, reports of lung toxicity associated with TNF-α antagonists have recently appeared, particularly in the rheumatology literature[5,6]. Our patient was exposed to two different biologics: ADA for 1 year and subsequently IFX for 6 mo. The pulmonary toxicity of ADA is controversial: there are reported cases of induced interstitial pneumonia[21,22] as well as reports of efficacy in the treatment of rheumatoid arthritis- and dermatomyositis-associated lung disease[21,23]. Our patient did not experience any respiratory symptoms during treatment with ADA, and she started complaining of dyspnea on exertion after the 5th dose of IFX. This timing is delayed compared with the previously reported experiences that occurred in the majority of rheumatology cases after the 2nd-3rd infusion[24-28]. Similarly, in the CD patient described by Weatherhead *et al*, symptoms appeared after the first infusion and were exacerbated after the 2nd[27]. In the most recently reported case of nonspecific interstitial pneumonia in a young female with UC, symptoms appeared after the 2nd infusion of IFX[30]. The timing of respiratory symptoms after the 5th infusion of IFX observed in our patient is similar to that reported by Wiener and colleagues in a 63-year-old woman affected by UC[31]. Most of the reported cases received anti-TNF α associated with other immunomodulators[6]; additionally, in the reports by Weatherhead and Wiener, the patients were taking other medications for IBD (azathioprine and balsalazide, respectively)[29,31]. Thus, it was hypothesized that TNF-α blocking agents might provide a favorable environment for the induction and/or the progression of iatrogenic lung disease through modulation of the immune system[32].

In the present case, it is reasonable to suspect drug-induced interstitial lung disease attributable to IFX for several reasons: (1) the onset of respiratory symptoms shortly after IFX introduction; (2) the 8-year treatment with mesalamine without any symptoms; and (3) the slow improvement after mesalamine discontinuation (despite its rapid wash-out period) and after IFX discontinuation (consistent with its long wash-out period). Given the seriousness of the adverse event, definite proof is unrealistic because a re-challenge with the drug would be unethical and dangerous; however, the lack of an anatomical diagnosis using open-lung biopsy limited the differential diagnosis.

In conclusion, there is emerging evidence that anti-TNF-α agents might induce lung toxicity even in the long term. High vigilance is recommended for the occurrence of respiratory symptoms in patients undergoing biological treatment.

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P- Reviewer D'Ovidio V **S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5381

World J Gastroenterol 2013 August 28; 19(32): 5381-5384
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Hepatotoxicity associated with glucosamine and chondroitin sulfate in patients with chronic liver disease

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Received: February 26, 2013 Revised: April 30, 2013
Accepted: June 5, 2013
Published online: August 28, 2013

Abstract

Glucosamine and chondroitin sulfate are molecules involved in the formation of articular cartilage and are frequently used for symptom relief in patients with arthrosis. These molecules are well tolerated with scarce secondary effects. Very few cases of possible hepatotoxicity due to these substances have been described. The aim of this paper is to report the frequency of presumed glucosamine hepatotoxicity in patients with liver disease. A questionnaire was given to 151 consecutive patients with chronic liver disease of different etiology (mean age 59 years, 56.9% women) attended in an outpatient clinic with the aim of evaluating the frequency of consumption of these drugs and determine whether their use coincided with a worsening in liver function test results. Twenty-three patients (15.2%) recognized having taken products containing glucosamine or chondroitin sulfate previously or at the time of the questionnaire. Review of the clinical records and liver function tests identified 2 patients presenting an elevation in aminotransferase values temporarily associated with glucosamine treatment; one of the cases simultaneously presented a skin rash attributed to the drug. Review of these two patients and the cases described in the literature suggest toxicity of glucosamine and chondroitin sulfate. The clinical spectrum is variable, and the mechanism of toxicity is not clear but may involve reactions of hypersensitivity. The consumption of products containing glucosamine and/or chondroitin sulfate is frequent among patients with chronic liver diseases and should be taken into account on the appearance of alterations in liver function tests not explained by the underlying disease.

Key words: Toxic hepatitis; Hepatotoxicity; Glucosamine; Chondroitin sulphate; Osteoarthritis

Core tip: A questionnaire was given to 151 consecutive patients with chronic liver disease of different etiology (mean age 59 years, 56.9% women) attended in an outpatient clinic with the aim of evaluating the frequency of consumption of these drugs and determine whether their use coincided with a worsening in liver function test results. Twenty-three patients (15.2%) recognized having taken products containing glucosamine or chondroitin sulfate previously or at the time of the questionnaire.

Cerda C, Bruguera M, Parés A. Hepatotoxicity associated with glucosamine and chondroitin sulfate in patients with chronic liver disease. *World J Gastroenterol* 2013; 19(32): 5381-5384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5381.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5381

INTRODUCTION

Glucosamine and chondroitin sulfate are precursor molecules involved in the synthesis of glycosaminoglycans

which make up the connective tissue. Their integrity is important to maintain the strength and elasticity of articular cartilage which confers resistance to mechanical stress[1]. In addition to its role as a substrate in the synthesis of cartilage and connective tissue, anti-inflammatory properties through the inhibition of the synthesis of nitric oxide have been attributed to glucosamine[2]. In relation to these functions, glucosamine and chondroitin sulfate have been used in the treatment of articular hyaline cartilage degeneration with the aim of stimulating the production of cartilaginous matrix[3].

Adverse effects from the exogenous administration of glucosamine and/or chondroitin sulfate are observed in less than 5% of the patients, with the most frequent being: gastrointestinal disturbances (discomfort/epigastric pain, pyrosis, diarrhea, nausea, and dyspepsia), somnolence, cutaneous reactions and headache. Two patients receiving glucosamine who were attended in our unit presented an unexplained elevation in transaminase values with no associated symptoms which reverted on discontinuation of the medication and was interpreted as a possible toxic hepatitis by this drug. We therefore decided to prospectively investigate the frequency of use of glucosamine and the incidence of elevations in aminotransferase which might be related to this drug. We studied patients with chronic liver disease since we considered that these patients may have limitations for the use of non steroidal anti-inflammatory drugs due to the risk of gastrointestinal bleeding or renal failure and may, thus, be a group in which the use of glucosamine or chondroitin sulfate is frequent.

CASE REPORT

From May 2011 to July 2011, 151 consecutive patients attended in an outpatient clinic for liver diseases by one of the authors were evaluated. All were asked about previous or current intake of drug products composed of glucosamine or chondroitin sulfate using a questionnaire including the name of all the commercial products containing these compounds on sale in Spain as well as those which can be obtained by Internet. None of the patients refused to answer the questionnaire. Patients who replied affirmatively with respect to the use of these products were asked about the date of treatment initiation and the duration of drug use. The clinical charts were thereafter reviewed to determine if there were hepatic biochemical alterations coinciding with the use of the drug.

During the period mentioned, 151 patients, ranging from 19 to 85 years of age (mean 59.2 years), were interviewed; 56.9% being women. The liver diseases were chronic hepatitis/hepatic cirrhosis due to hepatitis C virus (38.2%), autoimmune hepatitis (12%), chronic hepatitis/cirrhosis by hepatitis B virus (7.1%), alcoholic cirrhosis (4.9%) and Wilson disease and primary biliary cirrhosis (3.5%).

Twenty-three patients (15.2% of the total) acknowledged having consumed products containing glucosamine (6 patients), chondroitin sulfate (16 patients) or both (1 patient). Ten were receiving the drug at the time of the interview. In 21 out of the 23 patients it could not be established whether the liver had sustained drug-induced damage since no elevation in aminotransferase above the usual values was observed in association with the administration of glucosamine or chondroitin sulfate. A relationship between an elevation in transaminases and product consumption was detected in 2 cases, both of which had taken glucosamine. The first was a 71-year-old woman with chronic hepatitis C who had taken glucosamine sulfate during one year and presented an elevation in aminotransferases of 5 to 7-fold greater than the normal values during this treatment. The clinical records did not mention the use of other drugs. Viral infection due to hepatitis A, hepatitis B, and cytomegalovirus was excluded by serological tests. Serum transaminases returned to the usual values after treatment discontinuation. The second case was a 77-year-old woman with chronic hepatitis C who had taken glucosamine for 3 mo in 1977 and had presented an allergic cutaneous reaction attributed to the drug. She had not been treated with any other drug. At that time the transaminase values rose 4-fold above normal. In the follow-up liver tests taken in June 2011, a minimum elevation of alanine aminotransferase similar to previous analyses was observed. Neither of these two cases presented hyperbilirubinemia or changes in the biochemical indices of cholestasis and did not present either symptoms or decompensation of their liver disease. The details of the analyses performed during the episode compared with previous and posterior registries of each case are shown in Table 1.

DISCUSSION

To our knowledge this is the first report on the consumption of products containing glucosamine and/or chondroitin sulfate in patients with chronic liver disease. The frequent intake observed in the population studied is substantial (23 out of 150 patients, that is 15%), with 2 cases of possible toxicity among 23 patients who acknowledged current or past intake. This represents hepatic toxicity of almost 9% in patients with chronic liver disease reporting consumption of these drugs. The two cases with liver damage coinciding with the treatment had chronic hepatitis C.

Review of the literature has shown several cases of alleged hepatotoxicity by glucosamine and chondroitin sulfate (Table 2). In 2007 one case of hepatitis with elevations in alanine aminotransferase and total bilirubin of 6- and 10-fold, respectively above normal values was reported in a patient who had taken glucosamine at the therapeutic doses for 4 wk prior to presenting jaundice and pruritus[4]. Other etiologies were ruled out with a complete study of the patient, and liver biopsy showed findings compatible with drug-induced hepatitis. Another report identified 3 cases of probable hepatotoxicity. The first was a patient with severe cholestatic hepatitis who developed fulminant liver failure resulting in death after having taken glucosamine for 4 wk. The second case was a woman who had consumed a glucosamine/

Table 1 Results of the liver function tests in cases of hypertransaminasemia related to glucosamine consumption

	1-yr prior to consumption	During consumption	1-yr after consumption
AST (UI/L) (normal < 40)			
Case 1	25	182	71
Case 2	36	161	36
ALT (UI/L) (normal < 40)			
Case 1	33	282	85
Case 2	53	162	37
GGT (UI/L) (normal < 40)			
Case 1	32	150	77
Case 2	18	31	15
AP (U/L) (normal < 290)		229	219
Case 1	240	183	213
Case 2	144		
Total bilirubin (mg/dL) (normal < 1.2)			
Case 1	0.3	0.8	0.4
Case 2	0.8	0.9	0.9

The results of the two cases are compared with analyses performed 1-yr prior to and after the episode. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma Glutamyl transpeptidase; AP: Alkaline phosphatase.

chondroitin compound and presented anorexia, jaundice and cutaneous rash with persistence of biochemical alterations 6 mo after the onset of the symptoms. During the follow up the patient developed signs of hepatic dysfunction, and liver biopsy showed chronic portal hepatitis. The third case was a patient presenting an asymptomatic elevation in transaminases after having consumed a compound containing glucosamine for 6 mo. Complete recovery was achieved on discontinuation of the drug[5]. Two cases of probable hepatotoxicity were described in 2010 in relation to the consumption of a dietetic supplement (move free advanced) which contained glucosamine and chondroitin sulfate. The first of these cases presented diarrhea with an elevation in aminotransferases and alkaline phosphatase and the second showed a slight increase in aminotransferases with no specific symptoms. Neither case presented jaundice nor features of hepatic failure and the two patients improved 7 and 12 wk after withdrawal of the product[6].

The two cases attended by one of us (AP) were patients with no previous liver disease in whom a relationship was observed between the consumption of glucosamine and alterations in liver function tests. One was a 28-year-old woman who presented features of acute hepatitis with jaundice and pruritus at one month of starting treatment with glucosamine because of rough discomfort in both knees after minor trauma. Blood tests improved slightly on discontinuation of treatment with glucosamine with the subsequent disappearance of the symptoms. Viral, alcoholic, metabolic and autoimmune etiologies of the disease were ruled out. A liver biopsy performed one year later due to the persistence of biochemical alterations showed signs of chronic hepatitis. The other patient was a 56 year-old woman who had persistent transaminase values 3-fold greater than normal. All the potential causes of liver diseased were discarded and transaminase values normalized on withdrawal of glucosamine. The treatment was prescribed to improve initial symptoms of osteoarthrosis.

Taking our cases and those reported in the literature into account several characteristics may be pointed out. Firstly, all the cases had consumed compounds with glucosamine or chondroitin sulfate at the recommended therapeutic doses with no warning on the possible influence of doses within the range of the risk of hepatotoxicity. Neither was any other possible cause of liver damage identified. Suspicion of a toxic etiology in our cases was based on the infrequency of episodes of important elevations in transaminase values in chronic hepatitis C with no concomitant cause as well as regression on drug discontinuation. Jaundice was the most frequent initial symptom of hepatic compromise in the published cases but some cases presented asymptomatic alterations in liver biochemistries, one being severe hepatic failure and another developed chronic liver disease.

The mechanisms involved in the drug-induced hepatotoxicity are not clear. It is of note that the raw material used in compounds containing glucosamine are obtained from biopolymers of shells from marine invertebrates (shrimps, crabs, lobsters) and chondroitin sulfate is taken from cow trachea cartilage and shark cartilage in Japan[7]. One of our cases and two of those reported in the literature simultaneously presented hypersensitivity reactions and thus, hypersensitivity may have been the contributory mechanism, at least in one of the cases. Responsibility of additives contained in the glucosamine preparations used for our patients seems unlikely, because neither aspartame, sorbitol, citric acid or polietilenglicol have been related to liver injury.

Glucosamine is a precursor to glycosaminoglycan, which is believed to play a role in the growth of cartilage and its repair. Chondroitin is part of a large proteoglycan molecule that gives cartilage flexibility and is thought to inhibit enzymes that break down cartilage. Glucosamine is used in the treatment of osteoarthritis, a disease resulting from the articular hyaline cartilage degeneration which leads to the loss of cartilage. Osteoarthritis is very prevalent in general population, particularly in elder subjects. It causes significant morbidity due to pain and functional disability of joints, and increased health care costs as well[8]. The reason for the use of glucosamine or chondroitin sulfate in these patients lies in the belief that osteoarthritis is associated with a deficiency in key natural substances and these products provide a substrate for the synthesis of cartilaginous matrix. In addition, they provide protection against enzymes which degrade the cartilage[7]. Some randomized placebo-controlled trials using glucosamine showed a decrease of the symptoms of osteoarthritis in the group receiving glucosamine in comparison with the control group, but this not found in others[8-11]. No side effects related to the liver were observed in these trials. In the 2005 Cochrane review it was reported that in studies older and of lesser quality the effect of placebo was greater, while pain relief was

Table 2 Summary of the cases reported in the literature on hepatotoxicity by glucosamine and/or chondroitin sulfate

Ref.	Age (yr) Sex (F/M)	Drug consumed	Length of consumption	Latency	Jaundice	Peak in AST/ ALT (IU/L)	Hypersensitivity	Hepatic failure	Follow-up
Ossendza *et al*[4]	52/M	Glucosamine	3 wk	4 wk	Yes	263/63	Pruritus, eosinophilia	No	Complete recovery
Smith *et al*[5]	64/M	Glucosamine/ chondroitin sulfate	4 wk	5 wk	Yes	-/1461	-	Yes	Death
	57/F	Glucosamine	4 wk	5 wk	Yes	-/1130	Pruriginous rash	No	Chronic hepatitis
	55/F	Glucosamine	6 mo	8 mo	No	-/175	-	No	Complete recovery
Linnebur *et al*[6]	71/F	Glucosamine/ chondroitin sulfate	7 wk	3 wk	No	600-700/ 00-500	-	No	Complete recovery
	85/F	Glucosamine/ chondroitin sulfate	3 wk	3 wk	No	54/37	-	No	Complete recovery
Authors' cases	71/F	Glucosamine	1 yr	NA	No	182/282	-	No	Liver tests return to pretreatment (basal) values
	77/F	Glucosamine	3 mo	NA	No	161/162	Pruriginous rash	No	Liver tests return to (basa) values

M: Male; F: Female; AST/ALT: Aspartate aminotransferase/ alanine aminotransferase; NA: Not available.

similar in patients receiving glucosamine or placebo in studies of better quality[12]. In Europe the different compounds containing glucosamine or chondroitin alone or in combination require a medical prescription, but in North America they may be purchased as a supplement without prescription, thus adding an extra risk of potential adverse effects because the drugs are taken without any medical judgment or are poorly purified.

Mild forms of hepatotoxicity may remain undiagnosed because of the absence of clinical expression with laboratory analyses not being performed in patients complaining of joint pain before and during treatment with glucosamine or chondroitin sulfate. Our observations suggest that these products should be suspected as a possible cause for the analytical changes in patients receiving treatment with these drugs who show an alteration in transaminase values. In these cases, drug discontinuation seems justified taking into account their low or questionable therapeutic efficacy and the possibility of developing more severe liver damage with continued use.

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P- Reviewers Nakajima A, Philip A, Yukihiro S
S- Editor Zhai HH **L- Editor** A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5385

World J Gastroenterol 2013 August 28; 19(32): 5385-5388
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

A case of rapidly progressing leiomyosarcoma combined with squamous cell carcinoma in the esophagus

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Received: March 12, 2013 Revised: June 15, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Abstract

Esophageal leiomyosarcoma is a rare tumor that accounts for less than 1% of all malignant esophageal tumors. Esophageal leiomyosarcoma combined with squamous cell carcinoma is even rarer than solitary leiomyosarcoma. We experienced a case of leiomyosarcoma combined with squamous cell carcinoma that progressed very rapidly.

Key words: Leiomyosarcoma; Carcinoma; Squamous cell; Esophagus; Sarcoma

Core tip: We performed esophagectomy with esophagogastrostomy to resect the tumor. Pathological examination of the surgical specimen revealed that it was combined with squamous cell carcinoma. Combined carcinoma should therefore be considered when leiomyosarcoma shows rapid progression.

Jang SS, Kim WT, Ko BS, Kim EH, Kim JO, Park K, Lee SW. A case of rapidly progressing leiomyosarcoma combined with squamous cell carcinoma in the esophagus. *World J Gastroenterol* 2013; 19(32): 5385-5388 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5385.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5385

INTRODUCTION

Leiomyosarcoma of the esophagus is a rare malignant tumor, accounting for less than 1% of all malignant esophageal tumors[1-3]. Esophageal leiomyosarcoma combined with squamous cell carcinoma is even rarer than solitary leiomyosarcoma. Simultaneous esophageal leiomyosarcoma and squamous cell carcinoma were first described by Ovens *et al*[4] in 1951. Leiomyosarcomas are characterized by slow growth and late metastases, and hence have a better prognosis than squamous cell carcinomas of the esophagus[5,6]. However, we experienced a case of leiomyosarcoma combined with squamous cell carcinoma that progressed very rapidly. We report this case and review the literature.

CASE REPORT

A 72-year-old male visited to our hospital suffering from chest pain that had been present for 1 mo. The patient had been diagnosed with colon cancer and received laparoscopic surgery 1 year prior. The physical examination was unremarkable. He was admitted to the cardiology department, and received electrocardiogram and cardiac single photon emission computed tomography, but no cardiac problem was found. Endoscopic examination

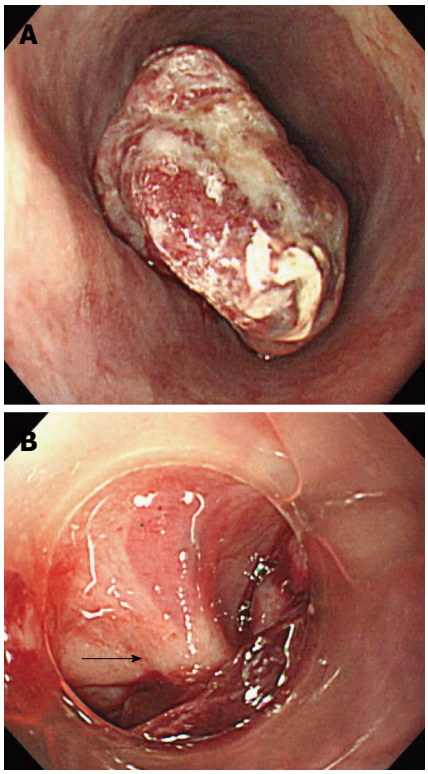

Figure 1 Endoscopic finding. A: Intraluminal polypoid mass; B: Stalk of the mass (arrow).

demonstrated a stalked intraluminal polypoid mass in the mid esophagus, 30 cm from the incisor (Figure 1). The tumor was large enough to fill the esophageal lumen, but allowed passage of a gastrofiberscope (Q260, Olympus, Tokyo, Japan) to the distal part of the esophagus. An endoscopic biopsy was performed, and the patient was suspected of leiomyoma and leiomyosarcoma. A computed tomography scan showed a large, well enhancing soft tissue mass in the mid esophagus (Figure 2), but no regional lymph node enlargement or liver metastasis. Positron emission tomography/computed tomography (PET-CT) showed intense segmental F-18 fluorodeoxyglucose (FDG) uptake [Standardized Uptake Value (SUV) max 17.3] at the mid-thoracic esophagus. Compared with the previous PET-CT for colon cancer follow-up from 3 mo prior, there was only physiologic FDG uptake at the esophagus (Figure 3). The patient underwent surgery; an esophagectomy with esophagogastrostomy. Macroscopically, the resected specimen was a polypoid tumor measuring 9.8 cm × 5.0 cm × 2.5 cm (Figure 4). Histopathologically, the tumor consisted of pleomorphic spindle cells with mitosis and cell necrosis compatible with leiomyosarcoma (Figure 5A). Tumor invasion involved the muscularis propria, submucosa, and mucosa. Nine regional lymph nodes were free of metastasis. An immunohistochemical examination stained positive for smooth muscle actin, but negative for cytokeratin and S-100 protein (Figure 5B). These were stained by an automated Ventana immunohistochemical/*in situ* hybridization staining platforms machine (BenchMark XT). Squamous

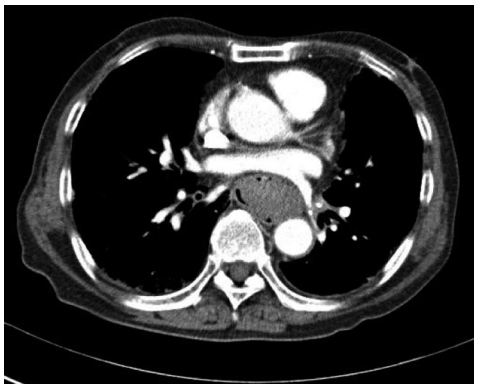

Figure 2 Computed tomography scan showed a large, homogeneously enhancing soft tissue mass.

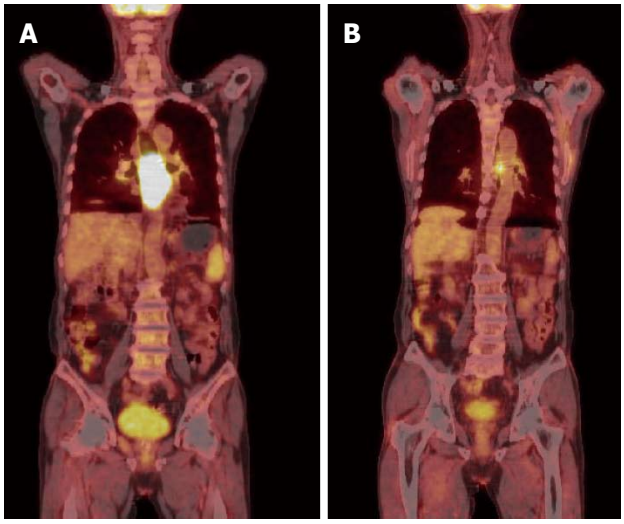

Figure 3 Positron emission tomography/computed tomography. A: Positron emission tomography/computed tomography (PET-CT) showed intense segmental fluorodeoxyglucose uptake (SUV max 17.3) at mid esophagus; B: PET-CT performed at 3 mo ago.

severe dysplasia and focal stratified squamous epithelial invasion into the lamina propria was also noted in the mucosa (Figure 5C). We diagnosed the patient with leiomyosarcoma combined with squamous cell carcinoma.

In the post-operative period, the patient recovered uneventfully and was discharged 18 d after operation. No adjuvant radiotherapy or chemotherapy was administered. At the last follow up visit to our hospital 2 mo after surgery, the patient was in good condition without any recurrence or distant metastasis.

DISCUSSION

Leiomyosarcoma is a high grade, smooth muscle soft tissue tumor that can occur in any tissue containing smooth muscle fibers. A leiomyosarcoma combined with squamous cell carcinoma is an extremely rare disease of the esophagus, with very few such cases described[4,5,7-9]. Leiomyosarcomas are most commonly located in the middle and lower thoracic esophagus because smooth muscle

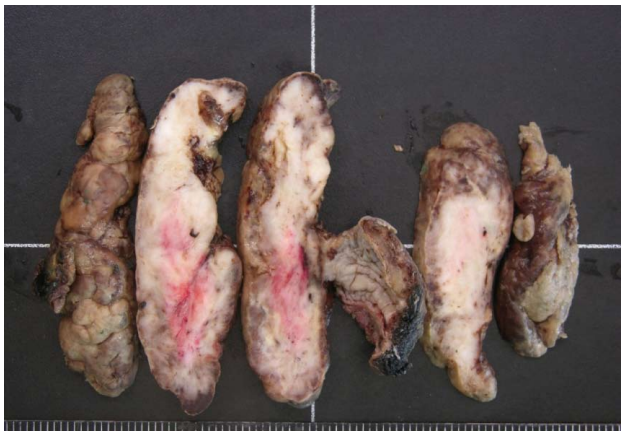

Figure 4 Resected specimen measured about 9.8 cm × 5.0 cm × 2.5 cm.

predominates in that area[10]. Esophageal leiomyosarcomas are typically divided into two types: the polypoid type in 60% of cases and the infiltrative type in 40% of cases[11,12]. Our case was the polypoid type. The prognosis of esophageal leiomyosarcoma is better than esophageal squamous cell carcinoma because of its characteristics of slow growth and late metastases[5,6]. Patients with polypoid and intramural tumors, tumors in an intrathoracic location, and well-differentiated tumors have a better prognosis than patients with infiltrating lesions, tumors in cervical locations, and poorly differentiated tumors[13,14]. Koga *et al*[13] reported a case of esophageal leiomyosarcoma that grew rapidly and had a poor prognosis. We think this case is unique because the tumor had good prognostic factors, such as the polypoid type and intrathoracic location, but it grew very rapidly and was combined with squamous cell carcinoma. Eroğlu *et al*[7] suggested that mutability or metaplasia between mesenchymal and epithelial tissues or multipotent stem cells with the ability to undergo biphasic differentiation toward mesenchymal and epithelial elements could be a mechanism of this combined malignancy. It is possible that these are separate entities that have arisen independently and combined squamous cell carcinoma may affect the growth of leiomyosarcoma by cytokines or growth factors.

The role of FDG-PET-CT in the diagnosis of leiomyosarcoma was reported recently[15-17]. Our case showed intense FDG uptake on PET-CT. The standard treatment is esophagectomy, but the role of adjuvant radiotherapy or chemotherapy is controversial with some authors[2,6,14,18]. In our case, the leiomyosarcoma grew exceptionally rapidly and was combined with squamous cell carcinoma, so further research will be needed to reveal the relationship between leiomyosarcoma and squamous cell carcinoma.

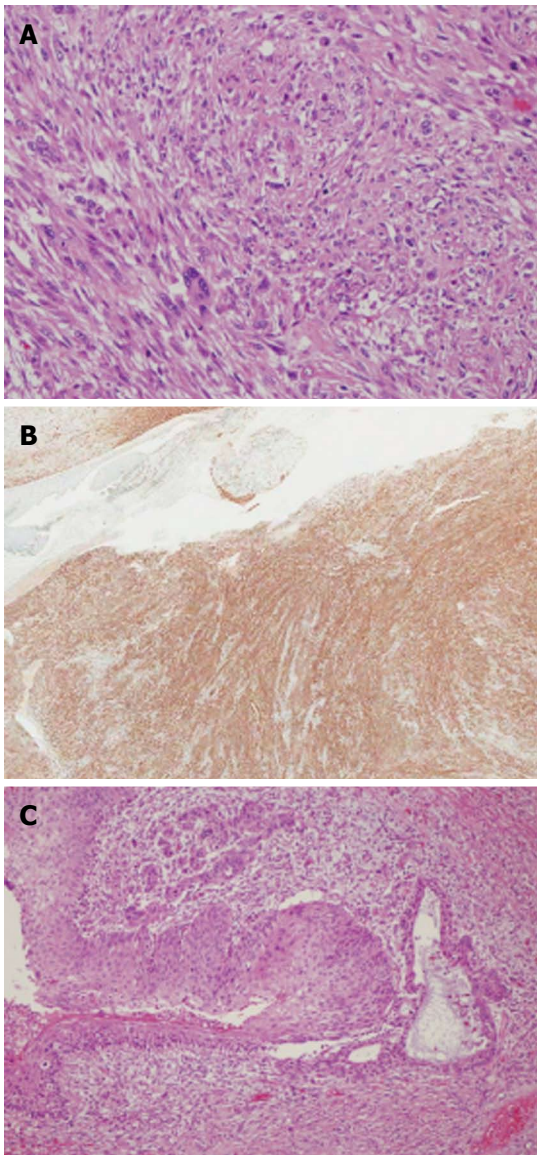

Figure 5 Pathologic images. A: Pleomorphic spindle cells showing mitosis and cell necrosis compatible with leiomyosarcoma [hematoxylin and eosin (HE) stain, × 200]; B: Immunohistochemical stain was positive for smooth muscle actin (× 12); C: Squamous severe dysplasia and focal stratified squamous epithelial invasion into lamina propria was noted in mucosa (HE stain, × 100).

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P- Reviewer Yeudall WA **S- Editor** Zhai HH
L- Editor Rutherford A **E- Editor** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i32.5389

World J Gastroenterol 2013 August 28; 19(32): 5389-5392
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Intestinal Behçet's disease appearing during treatment with adalimumab in a patient with ankylosing spondylitis

Sook Hee Chung, Soo Jung Park, Sung Pil Hong, Jae Hee Cheon, Tae Il Kim, Won Ho Kim

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Author contributions: Chung SH wrote the manuscript; Park SJ designed the research and performed peer review; Cheon JH analyzed the clinical data and consulted about pathologic data and medical agents; Hong SP, Kim TI and Kim WH reviewed the manuscript.

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Received: April 15, 2013 Revised: June 5, 2013
Accepted: July 18, 2013
Published online: August 28, 2013

Core tip: Here, we report on a patient who was diagnosed with intestinal Behçet's disease despite treatment with the fully humanized tumor necrosis factor-α blocker (adalimumab) for underlying ankylosing spondylitis. This patient achieved clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen.

Chung SH, Park SJ, Hong SP, Cheon JH, Kim TI, Kim WH. Intestinal Behçet's disease appearing during treatment with adalimumab in a patient with ankylosing spondylitis. *World J Gastroenterol* 2013; 19(32): 5389-5392 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5389.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5389

Abstract

Behçet's disease (BD) is a chronic inflammatory disease affecting multiple organ systems, such as the skin, joints, blood vessels, central nervous system, and gastrointestinal tract. Intestinal BD is characterized by intestinal ulcerations and gastrointestinal symptoms. The medical treatment of intestinal BD includes corticosteroids and immunosupressants. There have been several reports of tumor necrosis factor-α (TNF-α) blockers being successful in treatment of refractory intestinal BD. Here, we report on a patient who was diagnosed with intestinal BD despite treatment with the fully humanized TNF-α blocker (adalimumab) for underlying ankylosing spondylitis. This patient achieved clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen.

Key words: Intestinal Behçet's disease; Tumor necrosis factor-α; Adalimumab

INTRODUCTION

Behçet's disease (BD) involves multiple organ systems, such as the skin, joints, blood vessels, central nervous system, and gastrointestinal (GI) tract[1]. Intestinal BD is characterized by intestinal ulcerations and gastrointestinal symptoms[2]. The incidence of BD involving the GI tract varies by country, ranging from 3% to 60% of cases of BD[3]. GI bleeding and perforation can be associated with intestinal BD, with resultant comorbidities[1]. The medical treatment for intestinal BD includes corticosteroids and immunosupressants. Unfortunately, surgical treatment, such as ileocecal resection, is sometimes necessary for intestinal BD with perforation, intractable pain, and hemorrhage which are refractory to conventional therapy[4]. There have been several reports of tumor necrosis factor-α (TNF-α) blockers being successful in refractory intestinal BD. Most of these reported on the efficacy of infliximab[4-9] and a few reported on the efficacy of adalimumab[10,11]. Here, we report on a patient who was diagnosed with intestinal BD despite being treated with the fully humanized TNF-α blocker (adalimumab) for

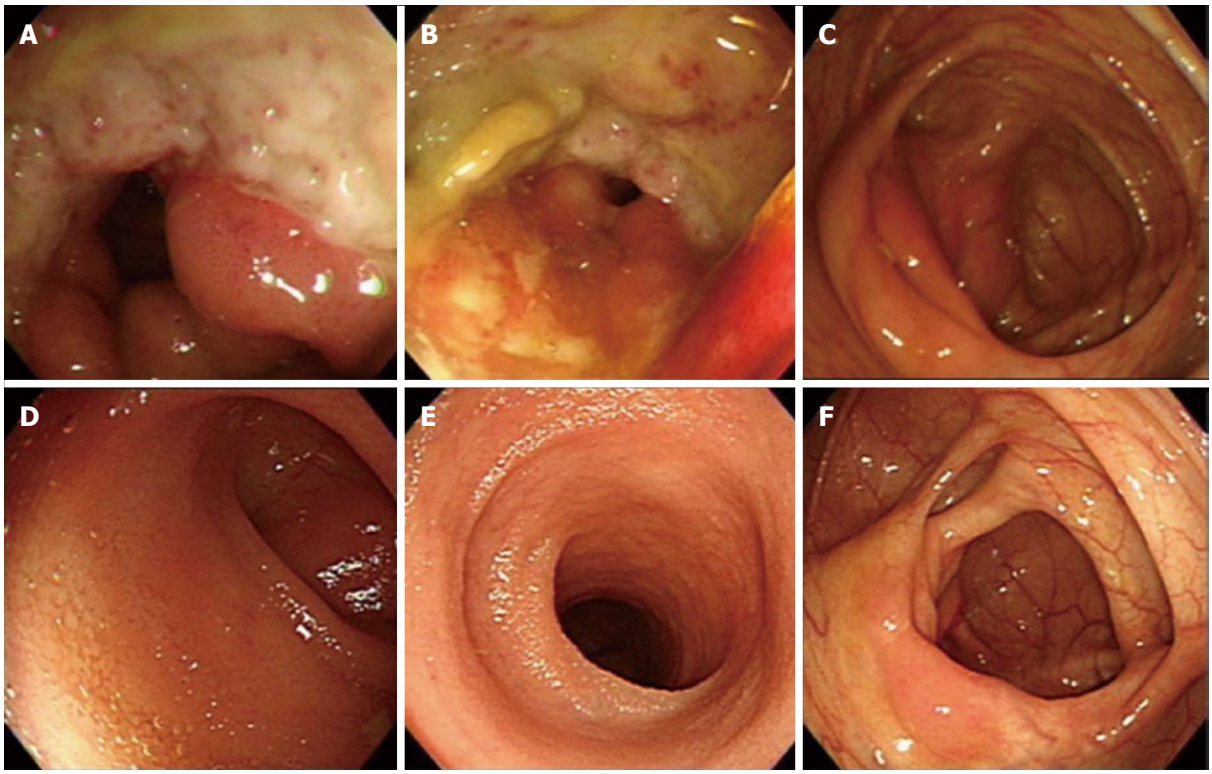

Figure 1 A colonoscopy on admission revealed a large, deep, well-demarcated ulcer with exudate, mucosal edema and erythema at the terminal ileum (A-C). On follow-up colonoscopy at 36 mo, the ulcer at the terminal ileum was replaced by normal mucosa (D-F) with complete mucosal healing.

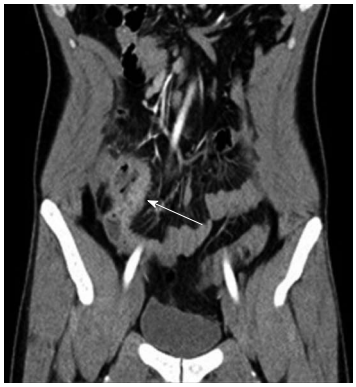

Figure 2 In computed tomography, arrow showed bowel wall thickening and prominent enhancement with surrounding fat infiltration at the terminal ileum and cecum. This was suggestive of active inflammation.

underlying ankylosing spondylitis. This patient achieved and maintained clinical remission and complete mucosal healing through the addition of a steroid and azathioprine to the adalimumab regimen for 43 mo.

CASE REPORT

A 29-year-old male patient was hospitalized due to severe right lower quadrant abdominal pain for the preceding 15 d. He had experienced recurrent oral ulcerations and arthralgia for 15 years and had had an erythematous papule on his back for the past 2 years. He had undergone appendectomy for appendicitis 17 years ago. He was diagnosed with ankylosing spondylitis 2 years ago because of lower back and shoulder pain. He had taken salazopyrine 1000 mg for 2 mo and had been injected with infliximab for his ankylosing spondylitis for 9 mo (5 mg/kg intravenously at 0, 2 and 6 wk; 5 mg/kg intravenously every 8 wk). The oral ulcerations, arthralgias, and erythematous papule on his back had improved, but his back pain had not been improved at that time. Therefore, the infliximab had been switched to adalimumab (40 mg subcutaneously every 2 wk) since 10 mo ago. On physical examination at admission, he appeared acutely ill, and had a blood pressure of 120/70 mmHg, a pulse of 84 beats/min, a respiratory rate of 24 breaths/min, and a temperature of 36.5 ℃. The abdomen was flat with direct tenderness in the right lower quadrant. Bowel sounds were normal. The results of laboratory tests showed a white blood cell count (WBC) of 20930/mm^3; hemoglobin, 13.9 g/dL; hematocrit, 41.7%; platelet count, 282000/mm^3; total protein, 7.2 g/dL; erythrocyte sedimentation rate increased to 33 mm/h; and C-reactive protein increased to 111 mg/L. A colonoscopy performed on admission showed a well-demarcated, large, deep ulcer with an exudate, mucosal edema, and erythema at the terminal ileum (Figure 1A-C). Colonic biopsies at the terminal ileum showed an ulcer with a necroinflammatory exudate. On computed tomography, bowel wall thickening and prominent enhancement with surrounding fat infiltration were noted at the terminal ileum and cecum, suggesting active inflammation (Figure 2). Finally he was diagnosed as intestinal BD according to the clinical symptoms and examination. The disease activity index for intestinal Behçet's disease (DAIBD) was 90, reflecting severe disease

Table 1 Clinical characteristics of patients with intestinal Behçet's disease receiving infliximab or adalimumab

Case	Age (yr)/ gender	Duration of disease (yr)	Anti TNF-α Ab for induction	Previous therapies	Maintenance therapy	Outcomes	Follow-up duration after achieving remission	Ref.
1	32/F	5	IFX	Steroids 6-MP	IFX 6-MP	Remission	9 mo	[14]
2	37/F	2	IFX	Mesalamine Steroids 6-MP	IFX 6-MP	Remission	16 mo	[14]
3	51/M	4	IFX	Steroids Methotrexate	IFX	Remission	3 yr	[14]
4	38/M	5	IFX	Steroids Colchicines Cyclosporine A	IFX	Remission	10 mo	[14]
5	43/F	6	IFX	Steroids Azathioprine	AZA	Surgery	6 mo	[14]
6	38/M	3	IFX	Steroids 6-MP	IFX 6-MP	Remission	2 yr	[14]
7	35/F	Over 20	IFX	Steroids Azathioprine	Methotrexate	Relapse	8 mo[1]	[7]
8	27 /F	2	IFX	Steroids Thalidomide	Steroids Thalidomide	Remission	17 mo	[5]
9	30 /F	3	IFX	Steroids Colchicines Cyclosporine	Thalidomide	Relapse	10 mo[1]	[5]
10	42/M	11	IFX	Steroids Colchicine	-	Remission	1 wk	[9]
11	47/M	20	IFX	Sulfasalazine Steroids Azathioprine	-	Remission	12 mo	[4]
12	30/F	-	IFX	Steroids Azathioprine	IFX Switch to adalimumab	Remission	22 mo	[10]
13	45/F	9	IFX	Mesalamine Steroids 6-MP	IFX	Remission	25 wk	[6]

[1]Duration between remission stage and the first relapse stage after infusion of anti tumor necrosis factor-α antibody (anti TNF-α Ab). F: Female; M: Male; IFX: Infliximab; 6-MP: 6-mercaptopurine.

activity[12]. Subsequently, the patient was treated with conventional medical therapy, including azathioprine 150 mg and 5-aminosalicylate (5-ASA, Pentasa) 3000 mg/d. His abdominal pain seemed to decrease after 10 d. However, the patient's severe right lower quadrant abdominal pain recurred after one month. The DAIBD score at the time of recurrent abdominal pain was 80, again reflecting severe disease activity[12]. In the early stages of treatment, clinical remission could not be obtained through combination therapy with azathioprine and 5-ASA. Thus, at the time of abdominal pain recurrence, intravenous hydrocortisone (300 mg/d) was administrated. Then the abdominal pain was improved 2 d after steroid injection. Intravenous hydrocortisone was slowly tapered to oral prednisolone for 2 mo. Finally, DAIBD score was 10. A follow-up colonoscopy after 36 mo demonstrated that the ulcer at the terminal ileum was replaced by normal mucosa (Figure 1D-F) with complete mucosal healing. Combination therapy with azathioprine, 5-ASA and adalimumab was continued for 43 mo with clinical and endoscopic remission.

DISCUSSION

We reported on a 29-year-old man with BD and ankylosing spondylitis who developed intestinal BD despite continuous use of adalimumab. We treated this patient with intravenous steroids to induce clinical remission and with azathioprine and 5-ASA for maintenance of remission. This case is unique in two ways. First, the terminal ileal ulcer characteristic of intestinal BD appeared while the patient was receiving adalimumab for ankylosing spondylitis. Second, mucosal healing was achieved and maintained through combination therapy with azathioprine and 5-ASA after induction of remission with intravenous steroids.

Conventional therapies such as mesalamine, corticosteroids, immunosuppressive agents, thalidomide, bowel rest, and total parenteral nutrition have been used in the treatment of intestinal BD[13]. However, in patients with intestinal BD unresponsive to conventional therapies, TNF-α blockers have been shown to improve symptoms[12]. Both infliximab and adalimumab can be used for treatment of intestinal BD because they are similar active biologics, monoclonal antibodies to TNF-α[10,11,14]. There has been no data available regarding the comparative efficacy of infliximab and adalimumab in intestinal BD. In Table 1 there have been many publications reporting on the effectiveness of infliximab[4-9]. However, there have been only a few reports of the efficacy of adalimumab

in treating intestinal BD[10,11]. Infliximab with combination therapies, such as 5-ASA and immunosuppressants, in patients with intestinal BD is effective for the induction and maintenance of remission[4-9,11,14]. In the case of adalimumab, adalimumab was reported to be effective in inducing complete remission as monotherapy[10]. We suggest three reasons why this patient may have developed an ulcer of the terminal ileum during the use of adalimumab, but not during the use of infliximab. First, the two medicines have different routes of injection, with adalimumab injected subcutaneously (SQ) and infliximab injected intravenously. When infliximab is injected intravenously, it enters the venous circulation directly with 100% bioavailability and no absorption phase, thereby reaching a more rapid therapeutic range than achieved with subcutaneous injection. Conversely, the bioavailability of an adalimumab 40 mg SQ dose has been estimated as 64%[15]. Second, there might be a difference in the effective dose between adalimumab and infliximab. Adalilmumab is used to be injected as fixed dose irrespective of body weight (40 mg subcutaneously every 2 wk) but infliximab is used to be injected according to the body weight (5 mg/kg at 0, 2 and 6 wk; 5 mg/kg every 8 wk). Compared to the infliximab, adalilmumab in fixed dose was supposed to be less effective in this patient with 22.8 kg/m^2 body mass index because of shortage of dose. Third, intravenous injection might be more potent than subcutaneous injection because BD is characterized by systemic vasculitis[16]. Infliximab and adalimumab are different medicines having unique pharmacokinetics. There was no head to head study for comparing effectiveness of infliximab and adalimumab. Further studies are warranted to compare the efficacy of adalimumab and infliximab in patients with intestinal BD.

In conclusion, this is the first case report of intestinal BD appearing despite the use of adalimumab. Furthermore, the subject in this case improved with the conventional combination of intravenous steroids for the induction of remission and azathioprine and 5-ASA for maintenance of remission. Despite the use of adalimumab, a conventional combination of therapies including intravenous steroids, azathioprine, and 5-ASA might be important in treating patients with intestinal BD.

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P- Reviewers Cuzzocrea S, Lukashevich IS **S- Editor** Zhai HH
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.3748/wjg.v19.i32.5393

World J Gastroenterol 2013 August 28; 19(32): 5393-5394
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Favorable effect of modest alcohol consumption to fatty liver disease

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Supported by Young Scientists (B) from Japan Society for the Promotion of Science, No. 23790791
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Received: May 3, 2013 Revised: July 9, 2013
Accepted: July 17, 2013
Published online: August 28, 2013

Abstract

We previously reported that modest alcohol consumption was significantly inversely associated with fatty liver disease. Feng *et al* pointed out a discrepancy of statistical significance between our current larger scale cohort and a previous cohort. However, the prevalence of non-alcoholic fatty liver disease was higher in non or minimal drinkers than those in light drinkers in both cohorts. They also argue that some potential co-factors such as soft drink consumption and genetic variations should be discussed.

Key words: Alcohol; Fatty liver disease; Obesity; Diabetes; Metabolic syndrome

Core tip: We reported the inversed association of modest alcohol consumption with fatty liver disease. However, other potential co-factors were argued to be important. Herein, we reply and discuss these important factors in this letter.

Hamaguchi M, Kojima T. Favorable effect of modest alcohol consumption to fatty liver disease. *World J Gastroenterol* 2013; 19(32): 5393-5394 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i32/5393.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i32.5393

TO THE EDITOR

Feng *et al* argued against the validity of the category of alcohol consumption we used, however this categorization was made in accordance with standardized methods[1,2]. They also pointed out the discrepancy of statistical significance between our current larger scale cohort and a previous cohort[3-5]. However, the prevalence of non-alcoholic fatty liver disease (NAFLD) was higher in non or minimal drinker than those in light drinker in both cohorts. In the current cohort, the prevalence of NAFLD was higher in non or minimal drinkers than those in light drinkers both in men (36.5%, 2248/6154 *vs* 26.4%, 457/1734) and in women (10.4%, 719/6893 *vs* 5.4%, 22/406)[4]. A similar trend was reported in the previous cohort as follows; the prevalence of NAFLD was higher in non or minimal drinkers than those in light drinkers both in men (28.6%, 170/595 *vs* 23.5%, 464/1977) and in women (10.7%, 105/981 *vs* 8.6%, 73/848)[5]. While statistical significance was not detected in the cohort used in our previous study, however we speculated that the smaller size of the cohort may have reduced the power of the statistical test and thus failed to detect the difference. Moreover, we previously reported that there is a favorable effect of modest alcohol consumption for the development of NAFLD in a prospective cohort study[6]. The adjusted odds ratio of light drinkers for the development of NAFLD was 0.82 (0.59-1.15, $P = 0.26$) in men, and 0.86 (0.51-1.45, $P = 0.56$) in women, respectively. The odds ratio was not statistically significant, but the

possibility of a favorable effect was speculated[6].

Feng *et al*[3] also argue that soft drink consumption or genetic variations may be potential co-factors for the favorable effect of modest alcohol consumption to fatty liver disease. Soft drink consumption is an important risk factor for NAFLD[7]. Unfortunately, in this case we had no data of soft drink consumption. However, a previous study has reported no association between alcohol consumption and soft drink consumption[8]. Thus, the association of NAFLD with soft drink consumption may be independent of those with alcohol consumption.

Genetic variations have been reported as important risk factors for NAFLD and non-alcoholic steatohepatitis[9-13]. The mechanisms of these genetic variations for development of NAFLD are still largely unknown. Thus, future investigations are needed; however, we appreciated Dr. Feng's extension of our discussion regarding the role of genetic variations in the pathophysiology of NAFLD.

In conclusion, we reported the inverted association of modest alcoholic consumption with fatty liver disease in a large scale cross-sectional study. Future large scale prospective studies and basic investigations are needed to clarify the effect of modest alcohol consumption in the development of fatty liver disease.

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P- Reviewers Dalamaga M, Pan Q, Tiribelli C, Wu JA
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Online Submissions: http://www.wjgnet.com/esps/
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World J Gastroenterol 2013 August 28; 19(32): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Launch date
October 1, 1995

Frequency
Weekly

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
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Room 903, Building D, Ocean International Center,
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Instructions to authors
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2012 Impact Factor: 2.547 (34/74 Gastroenterology and Hepatology).

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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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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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